FROST RESISTANCE IN *Eucalyptus nitens* (DEANE & MAIDEN). MAIDEN.

by

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STATEMENT OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other higher degree or graduate diploma in any tertiary institution, and to the best of the authors knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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ABSTRACT

Genetic and physiological aspects of variation in frost resistance were investigated in *Eucalyptus nitens* seedlings. Frost resistance was primarily determined by measuring the relative leakage of electrolytes from leaf discs subjected to artificial frosts, in an air-filled freezing chamber. The method used was a modification of that developed by other researchers with eucalypts. Frost resistance assessments made using leaf discs compared favourably with both artificially frosting whole seedlings and visual assessments of relative frost damage to field plantings subjected to natural frosts. Transmission electron microscope observations indicated that frosted leaf discs with a high relative leakage of cellular electrolytes, had cells that were characterised by rupture of the plasma membrane and tonoplast, and protoplasm coagulation.

The effects of photoperiod, temperature regime and differential root and shoot temperatures on frost resistance were examined in both hardening and dehardening *E.nitens* seedlings. Both hardening and dehardening processes were markedly influenced by temperature regime, but no photoperiodic responses were detected. Exposure to temperatures between 1.5 and 4°C for some part of the day/night cycle resulted in significantly increased frost resistance. Three separate experiments showed maximum hardening rates of *c.* 0.5°C $week^{-1}$. Increasing the daily amount of low temperature (2 to 3°C), from 8 to 16 h, resulted in increased levels of frost resistance. In contrast, seedlings exposed to constant 24 h low temperature, were almost 2°C less frost resistant than seedlings grown with 16 h day^{-1} low temperature after eight weeks, and suffered considerable water stress. Whilst heating seedling roots to between 6 and 18°C throughout the constant low air temperature regime maintained high plant water status, it did not confer any increased frost resistance. Rates of dehardening in well hardened seedlings increased with increasing day and/or night temperature in the range 6 to 20°C, but were significantly reduced if roots were kept at 3°C. The apparent quantitative response of both hardening and dehardening processes to temperature regime is discussed.

A comprehensive study of the genetic variation for frost resistance in *E.nitens* was made by means of artificial frostings, of both whole seedlings and leaf samples, and the assessment of damage from natural frosts to field trials. Two groups of provenances were identified. The Western provenance, from the Central Highlands of Victoria, and the provenance in Northern N.S.W. showed superior hardiness to the Southern N.S.W. and East Gippsland (Errinundra) provenances. This was demonstrated for plants that were unhardened, partially hardened and at near maximum hardiness. The critical frost temperature (50% leaf damage), separating the most and least hardy provenances, increased with the overall level of hardiness from only 0.3 to over 1.0°C, for unhardened and winter hardened plants respectively. At well developed levels of hardiness, individual families

hardened to below -10°C and differed by as much as 2.3°C. There were also significant trends of increasing frost resistance with altitude of seed source. The components of variance in frost resistance for "provenances" were about three times larger than components for "seedlings-within-provenance", for seedlings subjected to both artificial and natural frosts.

The early growth and frost resistance of *E.nitens* was compared with that of 12 other *Eucalyptus* species in trial plantations on two frost prone sites in Tasmania. Differences in levels of frost resistance amongst the species were over 7 and 3°C in winter and summer respectively. Species with the poorest frost resistance in winter, such as *E.fraxinoides*, *E.regnans* and *E.laevopinea*, suffered almost complete mortality at the coldest site. Only 16 months after planting there were highly significant differences in height and diameter growth amongst the species, with *E.nitens* by far the most productive. The possible interaction between growth rate and relative frost hardiness is discussed. Artificial frostings of reciprocal grafts between *E.nitens* and *E.gunnii* (a species with greater frost resistance), clearly demonstrated that levels of frost resistance in leaf tissue were largely determined by foliage genotype, irrespective of root stock genotype.

Intraspecific and interspecific controlled pollinations were attempted on a number of *E.globulus* ssp. *globulus* maternal parents. On the basis of capsule set, data indicate that *E.globulus* stigma are receptive a few days after anthesis, reach maximum receptivity after *c.* seven days and remain receptive for up to 12 days. Intraspecific and interspecific controlled pollinations were successfully made using a number of *E.nitens* maternal parents. Generally speaking the maternal influence was stronger than the paternal in terms of both number of seed set and seed weight. Controlled breeding methods in *E.nitens* and *E.globulus* ssp. *globulus* and some implications of the reproductive biology of *Eucalyptus* species for breeding programmes are highlighted.

Progeny from crosses onto *E.nitens* were morphologically intermediate with respect to both parents. The inheritance of morphological characters in eucalypts is discussed. The nature of the frost resistance of interspecific hybrids differed amongst the species combinations, sometimes appearing recessive or dominant but more commonly additive. *E.nitens X E.gunnii* seedlings from six different *E.nitens* mothers (representing the full range of provenances) all exhibited similar patterns in levels of frost resistance with respect to their parents. After only four weeks hardening *E.nitens X E.gunnii* seedlings were on average 1.3°C more frost resistant than their *E.nitens* mothers but only 0.5°C less frost resistant than their *E.gunnii* father. Frost resistance of intraspecific crosses generally displayed similar patterns (with respect to parents) to those of interspecific crosses.

Factors to consider when selecting and breeding for frost resistance in eucalypts are discussed.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Plant growth and development are affected by many biotic and abiotic factors. Amongst these environmental factors, the temperature regime to which the plant is exposed is critical for many processes. This is certainly so for the eucalypts (Paton 1980) where ,for example, seed germination (Grose 1963) and morphological development (Shepherd *et al.* 1976) are affected by the temperature regime. However, the range of temperatures suitable for satisfactory plant growth and development is restricted by both lower and upper limits, beyond which survival is ultimately threatened due to stressful conditions.

The effects of low temperatures on plants is of immense interest from both theoretical and practical viewpoints. For instance, it is estimated that world rice production would decrease by 45% if average world temperature fell by 1°C (Weiser 1982). However, to efficiently improve plant resistance to cold temperature stress, the fundamental issues of identifying the stresses and their repercussions, and determining the nature of molecular and cellular injury require greater understanding (Lyons *et al.* 1979).

There are two distinct types of low temperature stress, chilling stress and freezing stress (Levitt 1980). Chilling stress can be produced by temperatures between 0 and 12°C, which do not result in freezing of the tissue (Garber 1979). In this temperature range many species, particularly those of tropical or sub-tropical origin, suffer damage to, or impairment of, basic physiological mechanisms such as seed germination, growth, reproduction and post-harvest longevity (Lyons *et al.* 1979). Chilling injury is the term used to describe this disordered or impaired physiological functioning (dysfunction) at chilling temperatures. Many important crop plants, e.g. cotton (Sachs and Zilkah 1985), suffer chilling injury and are said to be "chilling sensitive" plants. In contrast, no references to chilling injury in *Eucalyptus* species could be found. Indeed, chilling temperatures are necessary to break physiological seed dormancy in many species of eucalypts, e.g. *Eucalyptus delegatensis* R.T.Baker (Grose 1963). In contrast, freezing stress is induced by sub-zero temperatures, although the temperature spectrum over which injury may occur is much broader being from -2 to <-70°C (see section 1.5 for more detail).

1.2 Freezing resistance terminology

An understanding of the nature of stress injury and resistance is a fundamental precursor to the examination and discussion of frost resistance in this study. When a plant is exposed to an unfavourable environmental factor, such as a frost, that factor acts as a stress

which may or may not produce a strain (Levitt 1980). The resultant strain is said to be elastic if it is reversible or plastic if it is irreversible (Levitt 1980). It therefore follows that there are two main types of resistance to stress, viz.,

a) elastic resistance, which is a measure of prevention of elastic or reversible strain, and

b) plastic resistance, which is a measure of prevention of plastic or irreversible strain.

Levitt (1980) points out that the level of resistance (R) to stress can be described as,

R = stress ÷ strain

Hence, resistance of a plant can be defined from this equation as the stress which results in a specific strain. Therefore, in terms of exposure to freezing, frost resistance can be defined as the stress (degrees Celsius) that is necessary to produce a given strain. One commonly chosen standard strain for determination of resistance is the point of 50% damage or death. Indeed, similar terms have been used in the study of frost resistance in eucalypts, viz., Harwood (1981) used the minimum temperature producing 50% leaf death (T_{50}), Menzies *et al.* (1981) used the temperature producing *c.* 30% foliage damage, and Hallam (1986) used the temperature producing 50% loss of cellular electrolytes (LT) from leaf tissue. In this study, assessment of relative frost resistance, particularly that using leaf discs (Chapters 4 to 7 and 10), is based on the frost temperature (stress °C) producing 50% damage (strain). Determination of relative frost resistance in planted trees subjected to natural frosts (Chapters 6 and 7), is somewhat different since comparisons are made of the different levels of damage (strain) after a given frost (stress).

1.3 Adaptations to freezing stress

Clearly, the mechanisms enabling plants to withstand and/or adapt to environmental stress cannot be overemphasised. Levitt (1980) suggests that two fundamental adaptations to freezing stress can be distinguished. The first kind of resistance adaptation is one in which thermodynamic equilibrium with the stress is avoided. In other words, the impact of a particular stress may be prevented by excluding or reducing its penetration into the plant. This type of resistance is called avoidance. Some plants may simply avoid freezing stress by avoiding sub-zero temperatures, either by growing in frost-free climates or by an ephemeral life cycle in mild seasons. However, it seems that avoidance of freezing temperatures whilst growing in frosty climates is largely unlikely, since plants are poikilotherms, i.e. they generally assume the temperature of their environment (Levitt 1980). Avoidance of freezing stress in some plants occurs due to supercooling, even beyond -20°C, e.g. woody deciduous species (George 1982) and *Rhododendron* (Kaku *et al.* 1982). Although supercooling to -7°C in eucalypts has been reported for *E.urnigera* Hook.f. (Thomas and Barber 1974) avoidance of freezing stress in eucalypts appears generally unlikely (Harwood 1980). It is basically excluded in controlled frosting studies where freezing is artificially initiated (Hallam 1986; Raymond *et al.* 1986). Freezing is also avoided in some plant tissues

because of their dehydrated state or the absence of intercellular space for extracellular ice formation (Ishikawa and Sakai 1982).

The second kind of freezing resistance adaptation is termed tolerance and is characterised by the ability of the plant to prevent, decrease, or repair any strain resulting from a stress. Hence, the plant tolerates the stress but may avoid or tolerate the implicated strain. The various avoidance and tolerance mechanisms of freezing resistance are summarized in Figure 1.1.

It appears that sometimes a number of terms are used somewhat synonymously when dealing with freezing resistance. The term "hardiness" is often used with plants, particularly as they harden as winter approaches and subsequently deharden (Chen and Li 1976), and has been used extensively in reference to eucalypts (Hunt and Zobel 1978; Griffin *et al.* 1982b; Hallam 1986). This term has a suggestion of robustness. The term frost tolerance has also been widely used in reference to eucalypts (Menzies *et al.* 1981; Raymond *et al.* 1986), and its usage would appear justified since freezing resistance is unlikely to be due to avoidance of ice formation. In this study the term frost resistance will be used since it encompasses both avoidance and tolerance adaptations. At times hardiness will be used, especially when dealing with hardening and/or dehardening, and relative survival and vigour of trees planted in frost-prone conditions.

1.4 Theory of freezing injury

The freezing process that occurs in plant cells and tissue when exposed to sub-zero temperatures was the subject of speculation and theorizing as early as the late seventeenth and early eighteenth centuries (Levitt 1980). Theories generally suggested that plant tissues expanded upon freezing and ultimately ruptured as a consequence. However, this was found to be unsound about 100 years ago when a number of scientists showed that plant cells actually contracted during freezing (Levitt 1980).

The physical and chemical events that take place as water from animal or plant cells freezes is now well described (see Mazur 1977). Basically, cells do not freeze at 0°C due to freezing point depression (which arises because of their constituent solutes) and supercooling. Freezing usually commences at temperatures around -2 to -15°C with ice nucleation, either on tissue surfaces or in the intercellular spaces between cells. The intracellular solution does not usually freeze upon ice nucleation in plants. Consequently, an unequal energy status (chemical water potential) arises between the water in the intracellular solution and that which is extracellular. The extracellular water, which is partially frozen, is characterised by a lower energy status (Lyons *et al.* 1979). Typically, the differential energy status between the two sites is brought into equilibrium by the movement of water from within the cells to the extracellular freezing sites. This results in cellular dehydration, the extent of which depends on the relative energy status of the intracellular solution (largely determined by osmolality) and the extracellular water (largely determined by minimum temperature). Alternatively, equilibrium may be achieved by intracellular ice formation, particularly where water efflux from cells is inadequate (Lyons *et al.* 1979). However, the

mass transfer mechanism operating with extracellular freezing generally occurs, since the heat transfer mechanism operating with intracellular freezing only occurs with excessive supercooling at rapid cooling rates, e.g. >3°C min^{-1} (Steponkus and Wiest 1979). Intracellular freezing is generally considered to be lethal (Levitt 1980).

The repercussions of the freezing process in biological systems are numerous, with membrane damage a widespread result and often implicated as the fundamental cause of injury (Lyons *et al.* 1979). Indeed, the leaves and stems of many plant species become flaccid and exhibit a water-soaked appearance following a frost-thaw cycle (Paton 1972), highlighting the pivotal importance of membranes in freezing injury. Results from nuclear magnetic resonance and electron spin resonance studies, strongly suggest that changes take place in cells at damaging temperatures whilst tissue is still frozen (Rajashekar *et al.* 1979).

Chilling and freezing injury are generally considered to be different, with freezing injury often manifested more rapidly than chilling injury (Rajashekar *et al.* 1979). Garber (1979) concluded from the responses of cucumber thylakoids to chilling, and spinach thylakoids to freezing, that there are differences in the stresses associated with chilling and freezing. Chilling injury appears to often involve biochemical or physiological dysfunction.

Mazur (1977) proposed that cells exposed to freezing temperatures are subjected to a series of events, any one or more of which may be potentially damaging. In such a system, it may be that injury does not act at one level but different cellular lesions arise as a result of different stresses. Some of the stresses associated with the decrease in temperature, presence of ice crystals and cellular dehydration, include a reduction in cellular volume, increase in solute concentration, changes in pH and the precipitation of buffering salts (Steponkus and Wiest 1979). The stresses associated with cellular volumetric changes on isolated protoplasts were addressed by Steponkus and Wiest (1979) who concluded that there were two resultant strains from a freeze-thaw cycle, i.e. "a freeze- or contraction-induced membrane alteration which decreases maximum critical surface area of the plasma membrane and a thaw- or expansion-induced dissolution of the plasma membrane which occurs when the maximum critical surface area is exceeded." Both strains interact with cell lysis a function of cell resilience, i.e. the capacity of the strained body to return to normal dimensions following stress.

Freezing and thawing of plants also appear to have several damaging effects on cellular reactions. Notably, early damage has been identified in mitochondria, chloroplast envelopes and stroma, and thylakoid membranes (Krause *et al.* 1982). Injury to active transport mechanisms of cell membranes is also implicated (Palta *et al.* 1977a,b). The breakdown of cell membrane properties with freezing injury and its associated efflux of ionic constituents and water infiltration, forms the basis of frost resistance determinations made by measuring the relative conductivity of effusate from frosted tissue in this study (see Chapter 4) and by other workers with eucalypts (Hallam 1986; Raymond *et al.* 1986).

Microscopic observations of freeze-injured potato cells, and in particular the examination of cellular ultrastructure, led Li *et al.* (1979) to suggest a possible sequence of freezing injury. Injury appeared to initiate with cell membrane abnormality leading to swelling of the protoplasm. With increasing stress, mitochondria and chloroplasts appeared

swollen. Finally, dead cells were characterised by separation of the tonoplast and plasma membrane (frost plasmolysis), and protoplasm coagulation.

1.5 Genetic and physiological variation in frost resistance

The level of frost resistance displayed in plants is dependent on two major factors,

a) the genetic ability of the organism to obtain specific level(s) of resistance to freezing, and

b) environmental factors which operate to bring about a specific level of resistance (Trunova 1982).

In other words, the characteristic level of frost resistance in plants is not necessarily static but is capable of varying under a host of environmental conditions, yet lies within a range of hardiness levels which is genetically determined. These two factors are discussed in turn.

There is a wide range in the freezing temperatures which cells and tissues are capable of resisting. At one extreme many deciduous hardwood species (Yoshie and Sakai 1982) and species from the genus *Pinus* (Oohata and Sakai 1982) are capable of resisting winter temperatures below -20°C, and some have even been uninjured at temperatures of -80°C (Pomeroy and Siminovitch 1971; Oohata and Sakai 1982). Even more extreme resistance has been reported for dehydrated (air dry) seeds, spores and pollen grains, although as Levitt (1980) points out, the lack of injury at temperatures as low as -190°C may be largely due to the inability of the samples to freeze because of lack of water. At the other extreme the cultivated potato *Solanum tuberosum* possesses little frost resistance, being injured at temperatures between -2 and -3°C (Chen *et al.* 1976). Generally, increased frost resistance is found in genotypes from environments exposed to colder conditions, either due to higher latitudes (Sakai *et al.* 1981; Oohata and Sakai 1982), higher altitudes (Guinon *et al.* 1982; Pryor 1957a) or frost hollows (Ashton 1958; Harwood 1980). Within this framework, levels of frost resistance in eucalypts range from approximately -3 to -20°C. The variation in frost resistance of eucalypts is discussed in detail in Chapter 2.

Great advances in productivity of plant species in given environments have been made through selection of genotypes for environmental adaptation, rather than some yield trait *per se*. This is certainly so for frost resistance in eucalypts, where generally the fast growing eucalypts of the Mediterranean climates fail in Atlantic climates due to frost damage and only the more frost resistant, albeit slower growing (on milder sites), species survive (Evans 1983). For example, *E.gunnii* Hook.f. is preferred in France, though it is generally slower growing than many other eucalypts (Potts and Potts 1986). Even on milder sites it is sometimes desirable to select for frost adaptation rather than wood production *per se*, e.g.the selection of the hardiest (though slower growing) provenances of *E.fastigata* Deane & Maiden in areas of New Zealand (Wilcox 1982c). Hence, genetic variation in frost resistance can be exploited in breeding programmes. Although breeding strategies face severe time constraints in long-rotation forest crops, they offer the potential of realising

a vital role in reducing losses and increasing yields in most plant species (Weiser 1982), e.g. root pruning seedlings in the nursery to increase their ability to withstand water stress (Bacon 1978), and use of a shelterwood to moderate environmental conditions experienced by regeneration (Leikola and Rikala 1983).

The level of frost resistance in plants also varies in response to environmental conditions. The ability of plants to increase in frost resistance when subjected to shortening daylengths and/or lowering temperatures, is termed cold acclimation or frost hardening (Kacperska-Palacz 1978; Chen and Li 1982). The later term is mostly used when referring to tree species, such as the eucalypts, and it will be used in conjunction with eucalypts in this study rather than the term cold acclimation. It would appear that hardening does not alter the mechanism of injury but only shifts the limit of tolerance to lower temperatures (see Krause *et al.* 1982).

The relative importance of daylength and low temperature in conferring hardening has been the focal point of studies with species from many genera, e.g. *Cornus* species (Fuchigami *et al.* 1982), *Eucalyptus* species (Eldridge 1969; Harwood 1980,1981; Paton 1981), *Pinus* species (Smit-Spinks *et al.* 1985), *Salix* species (Christersson 1982) and *Solanum* species (Chen and Li 1982). Many woody species in cool temperate climates harden in response to both shortening days and lowering temperatures during autumn and winter, and subsequently deharden with increasing photoperiod and temperatures in spring {e.g. *Quercus* species (Neilson and Wullstein 1980)}. However, photoperiod is generally not critically involved in the hardening process in crop species (Gusta *et al.* 1982) and eucalypts (Paton 1981) apart from the supply of photosynthates.

It would therefore appear that the process of hardening is under the control of a genetic system which is activated by environmental factors. Some species within a genus have been found incapable of hardening/acclimating even though other species harden/acclimate in response to low temperatures, e.g. *Solanun tuberosum* and *S.acaule* respectively. No reference to eucalypt species incapable of frost hardening could be found in the literature. Genetic differences in extents of hardening have been demonstrated for cereal crops, e.g. winter wheat (Gusta *et al.* 1982), coniferous trees, e.g. *Abies grandis* Lindl. (Kamíniski 1982), and evergreen trees, e.g. eucalypts (Rook *et al.* 1980). Some of the different hardening rates for plants are shown in Table 1.1.

To a lesser extent other environmental factors have been shown to affect the hardening response to varying degrees. In some species, the artificial imposition of a water deficit has led to increased hardiness (Chen *et al.* 1977a; Chen and Li 1977). Investigations into the effects of nutrition on hardening has produced conflicting results. Addition of nitrogen can decrease (Dexter 1933) or not affect (Pellett 1973) hardiness whilst addition of phosphorus and potassium can increase hardiness (Levitt 1980). In some eucalypt species waterlogging can result in reduced frost resistance (Davidson and Reid 1987). Salt stress sometimes leads to a reduction in frost resistance (Sucoff *et al.* 1976). However, these factors do not seem to have as marked an influence on hardening as low temperatures and/or shortening daylengths.

1.6 Mechanisms of freezing resistance

Changes in freezing resistance of many herbaceous and woody plants are accompanied by changes in plant morphology and/or physiological factors. Anatomical features (Chen *et al.* 1977b; Kezeli and Beridze 1982), sugars and starch (Chen and Li 1982), water content, growth regulators (Chen and Li 1982; Paton 1981,1982,1983), fatty, amino and nucleic acids (Guy and Carter 1984), and proteins and lipids (Willemot 1979; Kikvidze *et al.* 1982; Yoshida and Uemura 1984) have been variously linked with freezing resistance, though results are often conflicting. The relationship of some of these factors to freezing resistance is discussed in an informative review provided by Levitt (1980). Substances which accumulate during hardening and are implicated in conferring increased resistance to freezing, are termed cryoprotectants.

However, there have been few detailed studies of mechanisms of freezing resistance in eucalypts. Research has largely centred on investigations into the roles of growth regulators on levels of resistance, e.g. with *E.grandis* Hill ex Maiden. (Paton 1981, 1982, 1983) and *E.pulverulenta* Sims (Bowers 1983). In particular, the evidence available for *E.grandis* suggests that the content of the growth regulator, G, was associated with increased frost resistance and possibly operated through its effects on active transport properties of membranes (Paton 1981). Clearly, more detailed research into the physiological and biochemical mechanisms implicated in the frost resistance of eucalypts is warranted.

Following extensive studies on cold acclimation in potato (*Solanum*), Chen and Li (1982) developed a conceptual sequence of events indicating how environmental hardening signals were translated into biological adjustments which resulted in acclimation. They hypothesised that the resultant increase in sugars during cold acclimation (generally thought to be due to greater photosynthesis over respiration and growth) leads to an increase in cellular osmotic concentration which, upon reaching a critical level, triggers an elevation in ABA (abscisic acid) which induces protein synthesis. The synthesis of specific proteins appears largely responsible for resultant increases in freezing resistance through altered membrane properties. However, frost resistance mechanisms appear to be a complex interaction of many factors, with no one theory attracting widespread acceptance.

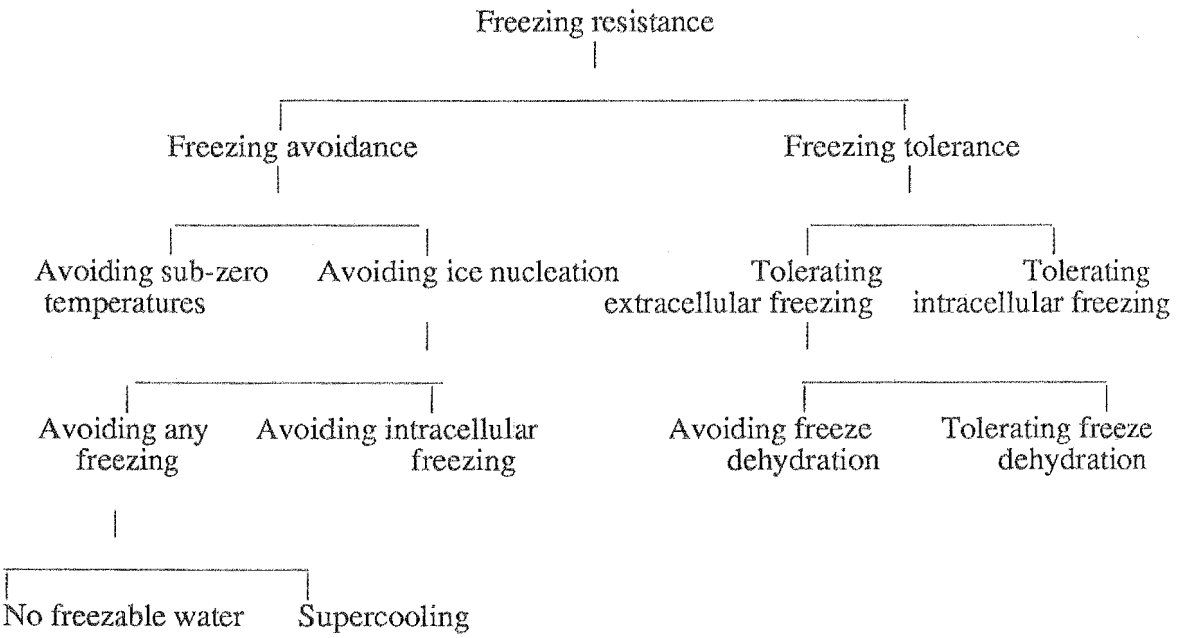


Figure 1.1 Freezing resistance adaptations. After Levitt (1980)

Table 1.1 Relative rates of frost hardening for various plant species.

Species	Unhardened frost resistance* (°C)	Treatment**	Hardening Time (days)	Hardening Rate (°C week^{-1})	Source***
Crop species					
Solanum acaule	-6.0	a	21	0.6	A
Solanum commersonii	-5.3	a	21	0.7	A
Solanum tuberosum	-3.0	a	21	0	A
Triticum spp.(cv. Norstar)	-3.0	b	21	6.6	B
Eucalyptus species					
Eucalyptus delegatensis	-4.4	c	56	0.5	C
Eucalyptus pauciflora	-4.5	d	78	1.7	D
Eucalyptus viminalis	-4.0	e	4	>4.0	E
Eucalyptus nitens	-3.2	f	56	0.5	F
Cool-temperate rainforest species (Tasmania)					
Nothofagus cunninghamii	-3.6	f	18	1.4	G
Atherosperma moschatum	-3.0	f	9	0.8	G
Phyllocladus asplenifolius	-3.4	f	9	1.6	G
Eucryphia lucida	-3.5	f	18	1.0	G
Atherotaxus selagenoides	-5.0	f	26	1.0	G
Cool temperate (northern hemisphere) species					
Pinus sylvestris	-8.5	g	42	>4.5	H

*:- temperature giving a standard level of damage

**:- day/night temperatures for treatments (a) 5°C (16 h)/0°C; (b) two days at each of 15/10°C, then 10/3°C, 10/1°C and 1/1°C; (c) 12°C (8 h)/0°C; (d) 18°C (10 h)/4°C; (e) 2°C constant (12 h photoperiod); (f) 25°C (16 h)/3°C; and (g) two weeks at each of 10°C (16 h)/3°C, then 3°C/0°C and 0°C/-3°C.

***:- A, Chen and Li (1976); B, Gusta *et al.* (1982); C, Hallam (1986); D, Harwood (1981); E, Paton (1981); F, this study (Chapter 5); G, Read (1985); H, Smit-Spinks *et al.* (1985).

CHAPTER 2

EFFECTS OF FROST ON *EUCALYPTUS* SPECIES

2.1 Geographic distribution of *Eucalyptus* species

The natural distribution of species from the genus *Eucalyptus* is largely confined to the Australasian region (Pryor 1976). By far the largest portion of the 500 or so recognised taxa (Chippendale and Wolf 1981) are endemic to Australia with only two species exotic to Australia, viz., Papua New Guinea and Timor (Pryor 1981; Turnbull 1981). Hence, the genus covers a wide latitudinal range, from 7° North to 43°South (Turnbull and Eldridge 1983). Within this range species can be found from sea level to altitudes of over 2000 m (Turnbull and Eldridge 1983), and on steep sites with good cold-air drainage to sites with impeded air drainage where cold air often accumulates (Harwood 1980). Therefore, *Eucalyptus* species naturally occur on a range of sites with varying degrees of exposure to frost. Some species are located in essentially frost-free areas whilst others are subjected to frequent and severe frosts, as low as -22°C (Hall *et al.* 1981; Davidson and Reid 1985). Therefore, the potential exists for a great range in adaptation to freezing stress. This has in fact been demonstrated and is discussed in Section 2.3.

The geographic distribution of *Eucalyptus* species has been extended through plantings in many overseas countries, as early as the first few decades after Australian settlement, e.g. Europe (Martin 1948), South Africa (Darrow 1984), and the U.S.A. (Groenendaal 1983). Interest in the genus has been increasing (Pryor 1978),largely because of the capacity of species to achieve fast and sometimes phenomenal growth rates under intensive plantation silviculture, e.g. 80 m^3 $annum^{-1}$ M.A.I. in Brazil (Camphinos 1980). However, although promising growth rates have been achieved on mild sites, the genus is not considered suitable in many overseas countries because of insufficient levels of frost resistance and a corresponding high risk of extensive mortality. This is particularly the case at latitudes exceeding 45°, such as in Britain (Evans 1983), France (Potts and Potts 1986), and Russia (Pilipenka 1969). The effects of frost on species from the genus *Eucalyptus* therefore have application in understanding natural distribution of species and the limit of their extension.

2.2 Damage to *Eucalyptus* species

2.2.1 Expression of damage

Frost damage to *Eucalyptus* species is usually manifest within a few days of frosting, whether subjected to artificial (Harwood 1980) or natural frosts (Paton 1972). Foliage damage is most noticeable and is usually characterised by a "coumarin-like" odour and the development of a flaccid, water-soaked appearance which gives way to necrosis and death (Paton 1972). Depending on the severity of frost, damage may be confined to small patches on leaves or complete death. Dead leaves may remain for some time (Bond 1945) but usually fall (Paton 1983). With severe damage all foliage may be killed, eventually resulting in complete defoliation. Buds and stems are also killed by frost though visual expression of damage may be slower than that in leaves (Ashton 1958). Stems often receive less damage than leaves indicating a slightly higher level of hardiness (Harwood 1980), whilst roots are damaged at milder temperatures than leaves indicating a lower level of hardiness (Cremer 1985). In many cases recovery takes place via epicormic buds on stems and branches (Martin 1948). However, severe frosts can kill stems to ground level (Pilipenka 1969; Potts and Potts 1986) or the whole individual (Pilipenka 1969).

2.2.2 Damage to natural populations

Natural populations of *Eucalyptus* species are occasionally damaged to varying extents following unusually severe frosts, and in some instances broad scale death of large trees and large areas of forest have been reported. In the early nineteenth century, extensive damage to large tracts of mature forest in the central highlands area of Tasmania was attributed to the intensive winter of 1837 (Calder 1850). In another instance, Bond (1945) reports that unusually severe winter frosts caused the death of large areas of six year-old *E.regnans* F.Muell., *E.nitens* (Deane & Maiden) Maiden and *E.delegatensis* regeneration (from the 1939 wildfires) on the Toorongo Plateau of Victoria. More recently, frost damage has been reported in Victorian forests (Paton 1983) and in naturally regenerating stands of subalpine *Eucalyptus* species in Tasmania (Davidson and Reid 1985).

It is interesting to note that all the instances cited above share two common characteristics. Firstly, damage patterns were strongly associated with topography, being most intense in localised gullies, swamps and depressions where cold air accumulation was favoured, giving rise to lower temperatures than surrounding higher ground. Secondly, damage was associated with severe frosts during winter, rather than out of season frosts. The severe frosts causally implicated in damage to natural populations occurred in June (Davidson and Reid 1985), July (Bond 1945) and August (Paton 1983). However, the occurrence of frosts in seasons other than winter, when plants are generally at lower levels of frost resistance (see Rook *et al.* 1980), are sometimes critical to both natural populations

(Thomas 1965) and field plantings (Ashton 1958; Davidson and Reid 1987). Bond (1945), Paton (1983) and Davidson and Reid (1985) all suggest that the occasional frost event of killing severity is likely to be one of the environmental factors limiting the growth and distribution of *Eucalyptus* species.

Snow falls are frequently associated with extremely cold weather. However, there are little published data on the effects of snow on *Eucalyptus* species. Snow can in fact have both damaging (Cremer 1983) and protective effects (Davidson and Reid 1985). Cremer (1983) reported that although large areas of natural forests are sometimes affected by snow fall, the overall level of damage is very slight (usually breakage of branches), in terms of percentage crown loss. However, individual trees may suffer extensive crown loss and/or die following uprooting. Some species may be more prone to stem and branch damage than others. In a two year old species trial in southern Tasmania *E.globulus* Labill. suffered more damage than four other species (C.L.Beadle pers. comm.). Conversely, small seedlings or portions of foliage can be protected from frost injury by the insulating effects of snow (Davidson and Reid 1985). More research in this area is needed.

2.2.3 Damage to plantations

Although responses of natural forests to frost have given insight to the effects of frost on *Eucalyptus* species, it is from many of the trial plantings that much valuable data have been obtained. Despite the fact that only small areas of *Eucalyptus* species have been planted in Australia (Tibbits 1986) relative to overseas, frost damage has been reported in a few trials, particularly those containing a range of genotypes planted in cold environments. McKimm and Flinn (1979) reported frost and snow damage were largely responsible for very low survival of *E.globulus* and *E.regnans*, compared with *E.nitens* and *E.delegatensis* planted on the frost-prone environment of the Toorongo Plateau in Victoria. They also noted better survival and growth where site preparation had effectively controlled vegetative cover, i.e. grass, bracken etc. However, the relative effects of vegetative cover on survival through modification of temperature microclimate and relative levels of competition could not be separated, viz., lower temperatures have been recorded where grass rather than bare soil is present (Grose 1960), and grass is generally considered vigorously competitive with planted eucalypt seedlings.

Considerable damage has also been reported in one of 12 Australian sites planted with an extensive trial containing 49 provenances of *E.regnans* (Griffin *et al.* 1982b). The patterns of variation in frost hardiness corresponded well with damage to similar provenances in New Zealand plantations, indicating little genotype by site interaction. Similarly, an *E.delegatensis* field trial (Batlow, N.S.W.) containing 64 provenances was damaged by frosts in the first two winters following planting (Boland and Dunn 1985). Again the relative ranking of provenances corresponded well with estimates obtained using artificial frosting of tissue samples collected from plantings in Tasmania and potted seedlings (Hallam 1986).

No evidence in the literature could be found of trials specifically established in

Australia to examine the effects of frost on *Eucalyptus* species. This is understandable since there is generally little interest in growing eucalypt plantations in Australia for wood production apart from Tasmania, and the main trait sought after is growth rate (see Cotterill *et al.* 1985; Tibbits 1986), rather than frost resistance *per se*. However, selection for frost resistance may become important where it is thought that more frost resistant genotypes of a desirable species could be planted on colder sites, rather than planting a more frost resistant species, whose other traits may be less desirable

It is quite understandable that there should be far more reports of frost damage to *Eucalyptus* species planted overseas, since vast areas have been planted in overseas countries, often in climatic regions periodically subjected to colder extremes than many of the natural environments of the species. Overseas plantings damaged by frost have provided much valuable data on many genetic and physiological aspects of frost resistance in the genus. However, as Martin (1948) points out, the accuracy of interpretations of relative frost resistance of *Eucalyptus* species is sometimes questionable because of two factors. Firstly, the trees used are not always pure species but show varying degrees of hybridization with other eucalypts. This is likely to be more widespread in plantings established with seed collected from mixed species plantings, where natural hybridization frequently occurs (see Pilipenka 1969; Potts and Potts 1986). Secondly, the differences amongst species cannot always be confidently stated because the material used may not come from the hardiest provenances. As Harwood (1976) also points out, interpretation of results is further restricted because the onset of frosty conditions is often preceeded by a period of warm weather, unlike the natural conditions experienced in Australia, and trees therefore may not have been able to develop a high level of frost resistance (see Meskimen 1983; Potts and Potts 1986). Nonetheless, data from overseas plantings do indicate the great variation amongst,and within,species that exists within the genus.

Frost damage to planted eucalypts has been reported in such countries as New Zealand (Rook *et al.* 1980; Wilcox 1982a,b,c), Argentina(Mendonza and Alliani 1983), U.S.A. (Hunt and Zobel 1978; Heavilin 1978; Meskimen 1983), Zimbabwe (Quaile and Mullin 1983), South Africa (Nixon 1977,1983; Darrow 1984), Portugal (Barreto 1983), Spain (Arias 1983), France (Potts and Potts 1986), Yugoslavia (Andonoski 1983), Russia (Pilipenka 1969), and the British Isles (Martin 1948; Evans 1983). In milder climatic regions (e.g. Mediterranean areas of Europe and the southern states of the U.S.A.),the more frost resistant species often show quite vigorous growth,and damage is largely confined to more sensitive species. However, in areas subjected to the harsher conditions of cold temperate Atlantic climates (see Evans 1983),many of the more frost resistant species survive and grow well for a number of years but are usually severely damaged as a result of exceptionally cold winters (temperatures below -20°C). Such cold spells have occurred a number of times over the past 100 or so years in Europe (see Martin 1948),and even trees 20 m or more tall have been killed to at least ground level. Evans (1983) concludes that although the genus holds much interest in the U.K. it is too much of a risk for widespread investment.

2.3 Genetic variation in frost resistance

2.3.1 Variation amongst species

Clear orders of frost resistance and sensitivity are often evident following frost damage to natural forests and mixed species plantations. Paton (1983) found the ranking of species in a natural Victorian forest damaged by winter frosts, from most to least resistant, was *E.pauciflora* Sieb. ex Spreng. > *E.viminalis* Labill. > *E.radiata* Sieb. High proportions and even large *E.radiata* trees were killed, whilst few *E.pauciflora* died. Likewise, five species in fully hardened, naturally regenerating pole stands, displayed differing degrees of sensitivity to severe winter frosts in Tasmania (Davidson and Reid 1985), viz., from most to least resistant *E.gunnii* > *E.coccifera* Hook.f. > *E.johnstonii* Maiden $\geq$ *E.delegatensis* >*E.pulchella* Desf. In each case, relative dominance of species was altered following frost damage and both Paton (1983) and Davidson and Reid (1985) suggest that severe natural frosts are an important environmental factor affecting species composition in frost-prone areas. The relative frost resistance of naturally regenerating subalpine eucalypt species obtained by Davidson and Reid (1985), generally agreed with that assessed following spring frost damage to plantings and artificial frostings of whole seedlings (Davidson and Reid 1987). However, rankings of some species were reversed with the spring frost or environmental growth factors such as waterlogging.

More information on the genetic variation in frost resistance within the genus is available from assessments of frost damage to plantings, particularly those containing species which are geographically isolated in their natural environment. Martin (1948) placed over 30 *Eucalyptus* species in frost resistance groups, based on the winter frost temperatures which species are just capable of resisting. Using similar category groupings, Evans (1983) presented a "cold hardiness classification" for about 30 of the more productive *Eucalyptus* species in the maritime cool temperate climatic region. Three main points can be drawn from their findings. Firstly, the most resistant species can survive short periods of -18 to -20°C but are unlikely to survive lower temperatures, and include *E.pauciflora* ssp. *debeuzevillei*, *E.pauciflora* ssp. *niphophila*, *E.parvifolia* Cambage and *E.gunnii*. These species are generally slower growing than many of the less frost resistant species. Secondly, some of the fastest growing *Eucalyptus* species with highly favourable wood qualities, e.g. *E.regnans* , *E.grandis* and *E.globulus*, possess low levels of frost resistance and are unlikely to survive temperatures of -9°C or lower for extended durations. Thirdly, there are a number of species which are capable of rapid growth, have favourable wood characteristics and possess intermediate levels of frost resistance. Species in this group may survive short periods of exposure to temperatures as low as -12 to -14°C and include *E.dalrympleana* Maiden, *E.delegatensis* and the species studied in this thesis, *E.nitens*. Similar rankings of species are found in countries with less severe climates {e.g. South Africa (Nixon 1983) and parts of the U.S.A. (Heavilin 1978)} than those where the basis of the classifications of Martin (1948) and Evans (1983) were formed.

Assessments of relative frost damage to plantings following frosts of known severity do not always indicate the full extent of differences between species. For instance, a number of species may be undamaged by a frost, which gives no indication of how much more resistant they were from those killed or from each other. Comparisons need to be made at a number of frost temperatures. The rankings of Martin (1948) and Evans (1983) overcome this to some extent,since they appear to be based on the collection of results from numerous frosts of varying severity.

Another approach used to elucidate the extent of differences amongst species is controlled freezing studies. Provided the results correlate well with field performance they can provide a quicker and cheaper method of separating species. Harwood (1980) artificially frosted autumn-hardened seedlings of four subalpine *Eucalyptus* species to -9.6 and -10.7, and -12.0°C, and estimated that the difference in temperature causing 50% leaf death between *E.dalrympleana* and *E.stellulata* Sieb. ex DC. (most resistant) was over 3°C. More frostings would have been required to more accurately predict species differences, but those obtained correlated well with the temperature microclimate of the habitats of each species, i.e. *E.stellulata* occupied colder sites. Menzies *et al.* (1981), by frosting whole seedlings of *E.saligna* Smith, *E.regnans* and *E.fastigata* Deane & Maiden, were able to deduce differences of 1.5°C and less amongst the species at various seasons. Their findings agreed well with field performance in New Zealand.

Recently, tissue samples have been frosted to determine relative frost resistance of *Eucalyptus* species (Sakai *et al.* 1981; Hallam 1986; Raymond *et al.* 1986). Hallam (1986) found differences in relative frost resistance amongst six species were not significant in early autumn but were highly significant in winter. Although site differences affected lethal temperature (temperature causing 50% leakage of cellular electrolytes from leaf discs), Hallam (1986) estimated that the relative order of species remained fairly similar across sites. For example, of the five species at the lowest elevation site (characterised by cold air accumulation), lethal temperatures in winter for single provenances of *E.delegatensis*, *E.nitens*, *E.globulus*, *E.grandis and E.regnans* were approximately -8.8, -8.2, -7.9, -7.2 and -7.0 respectively. Hence, artificial frosting studies have the capacity to quantify relatively small differences amongst species and can be used to highlight the effects of season, site, provenance and other genetic and/or physiological factors on variation in frost resistance.

2.3.2 Variation within species

Variations in levels of frost resistance within a species have been demonstrated for forest trees from many genera, e.g. *Abies grandis* (Kamínski 1982), *Sequoiadendron giganteum* (Lindley) Buchholz (Guinon *et al.* 1982), *Pinus sylvestris* L. (Kullman 1983). This aspect of variation in frost resistance has received particular attention in *Eucalyptus* species for many decades (e.g. Pryor 1957a). Indeed, the use of different provenances of various species in separate trials, and inherent provenance variation in frost resistance, is

sometimes used to explain apparent anomalous rankings of species between trials.

Detailed investigations of intraspecific variation in frost resistance have been undertaken in *E.pauciflora* (Pryor 1957a; Harwood 1980), *E.regnans* (Ashton 1958; Eldridge 1969; Rook *et al.* 1980; Wilcox *et al.* 1980; Wilcox 1982a,b), *E.fastigata* (Sherry and Pryor 1967; Wilcox 1982c), *E.viminalis* (Paton 1972; Jahromi 1983), *E.urnigera* (Thomas and Barber 1974), *E.gunnii* (Evans 1983) and *E.delegatensis* (Grose 1960; Boland and Dunn 1985; Hallam 1986). In all these studies, levels of frost resistance within species have been shown to generally increase with colder environmental conditions at the seed source, due to either higher latitudes, higher altitudes or frost hollows. These act as a warning and a guide, to those establishing trial eucalypt plantings on sites where frost may be a problem, to use a number of provenances of each species if possible, and preferrably select provenances from more frost-prone natural environments, where there may be increased adaptation to frost. The range in frost resistance within some species is often of the order of 2°C. In the case of *E.regnans*, maximum differences between provenances ranged from 1.5°C in spring to 2.5°C in winter (Rook *et al.* 1980). Hallam (1986) found that the most hardy and least hardy *E.delegatensis* provenances sampled, differed by a similar order of magnitude, viz., -8.6 and -6.0°C in winter. The provenances used in both studies generally comprised of collections from 10 to 14 open-pollinated trees from a single stand.

The components of variation among and within provenances were analysed for frost damage to *E.fastigata* planted in New Zealand (Wilcox 1982c). The great variation in seedlots was mostly explained in terms of variation amongst provenances, since within-provenance variation was only 33% of that between provenances. These results indicate that there is fundamental variation in frost resistance at both the provenance and family level. No information on the extent of differences (relative frost resistance in degrees Celcius) between families within a species could be found. It is conceivable that some differences greater than those between provenances should exist, although they may not exceed the 2.5°C difference between provenances by more than a degree or two. There also appears to be no published data on the differences between individual trees. However, clonal eucalypt plantations are established in France from selected individuals which are often chosen on the basis of superior frost hardiness, i.e. survival after a severe frost (Potts and Potts 1986). In the light of the reputed outstanding frost resistance of individual specimens (see Appendix 1, Evans 1983), more data should be collected on the patterns of variation in frost resistance at this level.

2.4 Breeding for frost resistance

The great deal of variation in frost resistance, both amongst and within *Eucalyptus* species, indicates that there is scope for selection and breeding of frost resistant genotypes. Considerable gains in productivity on frost-prone sites could be achieved, due to increased survival of more frost resistant species or provenances. Breeding for frost resistance alone is hardly likely, since breeders may wish to realise gains in production and utilisation traits such as M.A.I., stem straightness, wood quality, coppicing ability and rootability of

cuttings. Fortunately, frost resistance and early growth appear to be largely uncorrelated in *Eucalyptus* species, indicating the possibility of simultaneous selection for both traits, e.g. *E.camaldulensis* (Grunwald and Karschon 1977), *E.fastigata* (Wilcox 1982c) and *E.regnans* (Griffin *et al.* 1982b). Wilcox (1982b) also found that concurrent selection for a number of traits is possible in *E.regnans* families, including frost resistance, early growth, disease resistance {to *Micosphaerella nubilosa* (Cooke) Hansf.}, branching quality and stem straightness. This also may be so for other important *Eucalyptus* species, resulting in large gains in productivity.

There is clearly a great deal of interest in breeding eucalypts for frost resistance in those countries planting eucalypts where frost is a problem, as evidenced by the extent of participation in the "International Conference on Frost Resistance of Eucalypts" held in France 1983. Although many of the breeding efforts appear to be at the first stage of identifying the correct choice of species (e.g. Nixon 1983), some are at the next main stage of choosing most appropriate provenances (e.g. Darrow 1984). There appear to be few programmes which are at more advanced stages, i.e. selecting better families and/or individuals and using controlled breeding and/or vegetative propagation techniques (see Pryor 1985). Some advances have been made in this area in France where frost resistance is critical (see Potts and Potts 1986).

Actual gains in frost resistance through breeding programmes are not well documented for *Eucalyptus* species. However, in a *E.grandis* breeding programme in Florida, U.S.A., significant gains were made in "freeze tolerance" as well as height, stem volume and stem straightness (Meskimen 1983). Although the breeding strategy implemented differed from usual strategies (see Meskimen for details), some gains in frost resistance were realised at each generation. However, Meskimen (1983) believes that improvements in frost resistance were largely due to advanced generations growing faster and avoiding lethal temperatures in a cold inversion layer close to ground level, rather than selection for physiological attributes conferring frost resistance.

CHAPTER 3

E.NITENS

3.1 Taxonomy of *E.nitens*

The most recent classification, albeit informal, of the genus *Eucalyptus* is that of Pryor and Johnson (1971). Their classification considers that all of the eucalypts constitute a single genus, itself comprised of seven subgenera. Within subgenera, additional categories in descending rank are section, series, {subseries}, {superspecies}, species and {subspecies}. Those categories bracketed are not considered obligatory. Currently, there are moves to formally divide the eucalypts into a number of genera (Johnson and Briggs 1983).

In this classification, *E.nitens* is included in subgenus *Symphyomyrtus,* section *Maidenaria,* series *Viminales* and subseries *Globulinae.* Other species in this subseries group include, the Southern Blue Gums (superspecies *Globulus*), *E.cypellocarpa* and those in superspecies *Goniocalyx.*

3.2 Distribution of *E.nitens*

E.nitens, shining gum, is a fast growing species with a discontinuous distribution in the mountain ranges of New South Wales and Victoria. It occurs as far north as 30°S, in and around the New England National Park, and as far south as 38°S, at Mt. Erica in the Baw Baw Ranges; covering an altitudinal range from 600 m in Victoria to 1500 m on the tableland escarpments of Northern N.S.W. However, within this range the species is generally confined to small stands of isolated occurrence, except in some areas of Victoria, i.e. Errinundra and Toorongo (see below). Pederick (1979) identified four main areas of occurrence, viz., Northern N.S.W., Southern N.S.W., East Gippsland and the Central Highlands of Victoria (see Figure 3.1). From these regions he designated six main provenances as, Northern N.S.W., Southern N.S.W., Errinundra, Rubicon, Toorongo, and Macalister. The last three provenances are phenotypically similar and were grouped by Pederick (1979) into the Western provenance (from the Central Highlands of Victoria).

Throughout its range, *E.nitens* frequently occurs in mixed stands with various other *Eucalyptus* species. In the Western provenance, at altitudes of about 1000 m or more, *E.regnans* and *E.delegatensis* are the main associate species, whilst associations with *E.cypellocarpa* L.Johnson and *E.viminalis* are most common at lower altitudes. Within the Errinundra provenance *E.nitens* stands are often characterised by a well developed rainforest

understory, unlike most of the other areas in Victoria, and are generally associated with *E.fastigata*, *E.ovata* Labill., *E.viminalis* and *E.cypellocarpa*. In Southern N.S.W., the provenance where the range in altitude is greatest (*c.* 750 to 1450m), *E.nitens* is associated with a greater range of species. The most common are, *E.pauciflora* and *E.dalrympleana* at higher altitudes, and *E.fastigata*, *E.cypellocarpa*, *E.viminalis*, *E.obliqua* L'Herit., *E.fraxinoides* Deane &Maid., *E.elata* Dehnh. and *E.rubida* Deane &Maiden at lower altitudes. The populations in Northern N.S.W. are almost always characterised by associations with *E.pauciflora* and/or *E.dalrympleana*.

Temperature and rainfall records from meterological stations, located near stands of shining gum, are presented in Table 3.1. Shining gum is confined to regions with a mean annual rainfall of at least 700mm (and presumably in many areas well over 1000mm), mean maximum and minimum temperatures of about 17 and 4°C respectively, frequent frosts and often snow (Pearce *et al.* 1983). Official records of the Seed Centre, CSIRO Division of Forest Research, show that throughout its range *E.nitens* often occurs on loamy soils with a pH of 4.5 to 6.

3.3 Natural variation in *E.nitens*

Investigations into the genetic variation within *E.nitens* were initiated by the State Forests and Land Service of the Department of Conservation, Forests and Lands, Victoria (formerly Forests Commission of Victoria), as early as 1970 (Pederick 1985). A number of trials gave similar results, and indicate the existence of two distinct forms of the species.

One form, termed the "juvenile-persistent form" (Pederick 1979), has a wide distribution and occurs at both extremes of the natural range, viz., Northern N.S.W. and the Central Highlands of Victoria. Its foliage is characterised by the presence of a highly fluorescent polyphenol, in both its broad juvenile foliage and its adult foliage (Pederick and Lennox 1979). The transition to adult foliage usually takes place from two to four years after planting and the adult foliage is characterised by smooth margins. In contrast, the "early-adult form" is largely confined to the Errinundra provenance and its transition to adult foliage usually commences only one year after planting. The "early-adult form" has juvenile foliage that is narrow and only slightly glaucous, and adult foliage that is characterised by denticulate margins with gland-like structures. The highly fluorescent polyphenols, found in the "juvenile-persistent form", are absent in the "early-adult form". Overall, the "juvenile-persistent form" is the faster growing in both Victoria, at least to 12 years of age (Pederick 1985), South Australia (Cotterill *et al.* 1985), and Tasmania (Tibbits 1986). Observations made during controlled pollination studies and from Herbaria collections (see Table 8.4), indicate that the two forms also differ in flower and capsule morphology. The relative difference, in some of the characters, between the two forms are summarised in Table 3.2.

Pederick (1979) suggests that populations on the Errinundra plateau might have arisen as a consequence of introgressive hybridization, with *E.quadrangulata* Deane & Maid., appearing as the most likely donor species. *E.quadrangulata* is in the same series as

E.nitens , i.e. *Viminales* (Pryor and Johnson 1971), and it has square stems and marginal glands on the adult leaves (Brooker and Kleinig 1983).

Of considerable interest is the occurrence of a few, small, isolated stands of *E.nitens* with characters similar to those of the Errinundra provenance, located in the Toorongo provenance. These stands are about 250 km from the Errinundra plateau. Personal observations of some plantations established in Tasmania from routine seed collections of the State Forests and Lands Service of Victoria , indicate that the "early-adult form" may also occur in stands in the south of the Rubicon provenance, near Marysville. The origin of these isolated populations with the Errinundra-type genes is unclear. Pederick (1979), suggested that they may have become established, either as a result of migration from the Errinundra plateau or as a result of a separate yet independent event of introgression.

The pattern of populations in the Toorongo provenance with Errinundra-type characters is particularly interesting. A stand at Christmas Creek, in the south-west of the Toorongo provenance, is most like the Errinundra provenance, on the basis of the frequency of trees without the fluorescent polyphenol (Pederick and Lennox 1979). Twelve kilometres to the north-west of the Christmas Creek population is the Tanjil Bren population, which has a smaller proportion of trees without the polyphenol, viz., 50% at Tanjil Bren c.f. 86% at Christmas Creek, and 100% on the Errinundra plateau. At Mt. Toorongo, which is 16 km north-west of the Christmas Creek stand, an even smaller proportion of the trees do not have the polyphenol, i.e. 42%.

Pederick (1979) suggests that this gradient in the frequency of the Errinundra-like characters indicates that the gene flow is in a north-westerly direction from Christmas Creek. The Errinundra-type characters appear to have also spread to some parts of the Toorongo plateau, north of Mt. Toorongo , viz., near the Toorongo River and the Upper Thompson area (Pederick, 1985). In marked contrast to this apparent north-westerly flow of the Errinundra-type genes, is the absence of trees exhibiting any Errinundra-type characters in the Mt. Erica population, only 5 km east of Christmas Creek. Therefore, it appears that some factor is preventing the flow of the "early-adult form" in an easterly direction from Christmas Creek (Pederick 1979). The Mt. Erica and Christmas Creek populations are separated by the higher country of the Baw Baw ranges. However, the stands may not be completely isolated reproductively, as gene flow from trees with the "juvenile-persistent form" at Mt. Erica may take place in a westerly direction to the Christmas Creek stand. These patterns of gene flow in the Toorongo provenance are the subject of a study, currently underway at the University of Melbourne (I.Cook and I.S.Ferguson pers. comm.). Alternatively, the distribution of populations with the "early-adult form" may reflect the relative competitive ability of this form in specific environments. Observations made during field trips to natural shining gum forests suggest that the Christmas Creek stand is similar to the Errinundra stands in as much as both occur with a well developed rainforest understory. A rainforest understory is almost always absent in stands with the "juvenile-persistent form".

3.4 The significance of *E.nitens*

Natural stands of all provenances of *E.nitens* have been harvested for timber production. However, it is a relatively minor species, except on the Errinundra and Toorongo plateaus (Pederick 1979), and in some areas of Southern N.S.W., where it has been harvested for pulpwood. This is largely due to the small and scattered nature of the typical stands, and often its co-occurrence with species that have been traditionally favoured by the sawmilling industry, e.g. *E.delegatensis* and *E.regnans*.

However, shining gum is becoming an increasingly important species for wood production in Australia. Although small areas have been planted with *E.nitens* in Victoria (McKimm and Flinn 1979; Pederick 1979), and Southern N.S.W. (N.S.W. Forestry Commission pers. comm.), the largest plantings have taken place in Tasmania, the state with the largest current eucalypt plantation programme (Tibbits 1986). To the end of 1984, approximately 4000 ha had been planted with *E.nitens* in Tasmania, and current rates are at least 1500 ha annum^{-1}. Shining gum has become increasingly important in Tasmanian eucalypt plantations because of its rapid early growth and a high level of frost resistance (Tibbits 1986), relative to other fast growing *Eucalyptus* species (see also Hallam 1986).

3.5 Rationale for this study

The successful establishment of eucalypt plantations, and their ability to survive and produce utilizable products, is largely affected by a range of biotic and abiotic agencies. Environmental agencies such as, pathogenic fungi (Heather and Griffin 1978), insect pests (Carne and Taylor 1978; de Little 1983), grazing animals (Mc Ilroy 1978), fire (McArthur 1978) and climatic extremes (Paton 1980), are potentially injurious to seedling health and vigour. Of the climatic extremes, low temperature, and in particular sub-freezing temperatures, have been shown to be a major factor limiting the growth and distribution of *Eucalyptus* species in Australia (Harwood 1983; Paton 1983) and particularly Tasmania (Davidson and Reid 1985).

The increasing annual planting rate of eucalypt plantations in Tasmania (Tibbits 1986) has meant that more marginal sites, particularly colder sites at higher altitudes, are being planted. These plantations are subject to a greater risk of frost damage. Tasmania is one of the states most subject to freezing temperatures (Turnbull and Eldridge 1983). Record low screen temperatures of -7°C and lower have been recorded for half of the Tasmanian meteorological stations reported by Hall *et al.* (1981).

Severe frost damage has occurred (personal observations) in young *E.nitens* plantations at altitudes of *c.* 600m in Tasmania, following a cold spell in June 1983, when Davidson and Reid (1985) recorded a minimum temperature of -22°C. Though many of the damaged trees appear to have recovered via epicormic buds, their subsequent form and growth may be less than desirable when compared with undamaged trees. Frost damage has also been observed, in young *E.nitens* plantations, following light frosts (*c.* -3°C) in

mid-summer (near Scottsdale ,Tasmania), and also in late spring, to new plantings by the Victorian State Forest and Lands Service, on the Toorongo Plateau.

However there are little published data on relative frost resistance within *E.nitens*. Frost damage to a young trial in Natal, South Africa, revealed that most damage was recorded in those families which had a high percentage of adult leaves and a correspondingly low percentage of juvenile leaves (Nixon 1977). There is clearly a need to identify the most frost resistant provenances of *E.nitens*. This is important as most of the organisations planting *E.nitens* use seed from preferred populations. In Tasmania, seedlings from the Toorongo provenance (avoiding the "early-adult form") are planted because of superior growth (Tibbits 1986), and the N.S.W. provenances are preferred in South Africa (Darrow 1984).

3.6 The scope of this study

The scope of this study is to examine some of the genetic and physiological aspects of variation in frost resistance, within *E.nitens*. These are compared with those in other *Eucalyptus* species. The physiogical basis of frost resistance is investigated with particular reference to factors affecting hardening and dehardening. Some of the ultrastructural factors, which may be related to frost resistance, are also examined. Investigations into genetic variation are used to determine the most frost resistant provenance(s) and families, at a range of levels of hardiness. Intraspecific and interspecific controlled pollinations are made to determine the relative gains in frost resistance, which may be made from breeding with *E.nitens*.

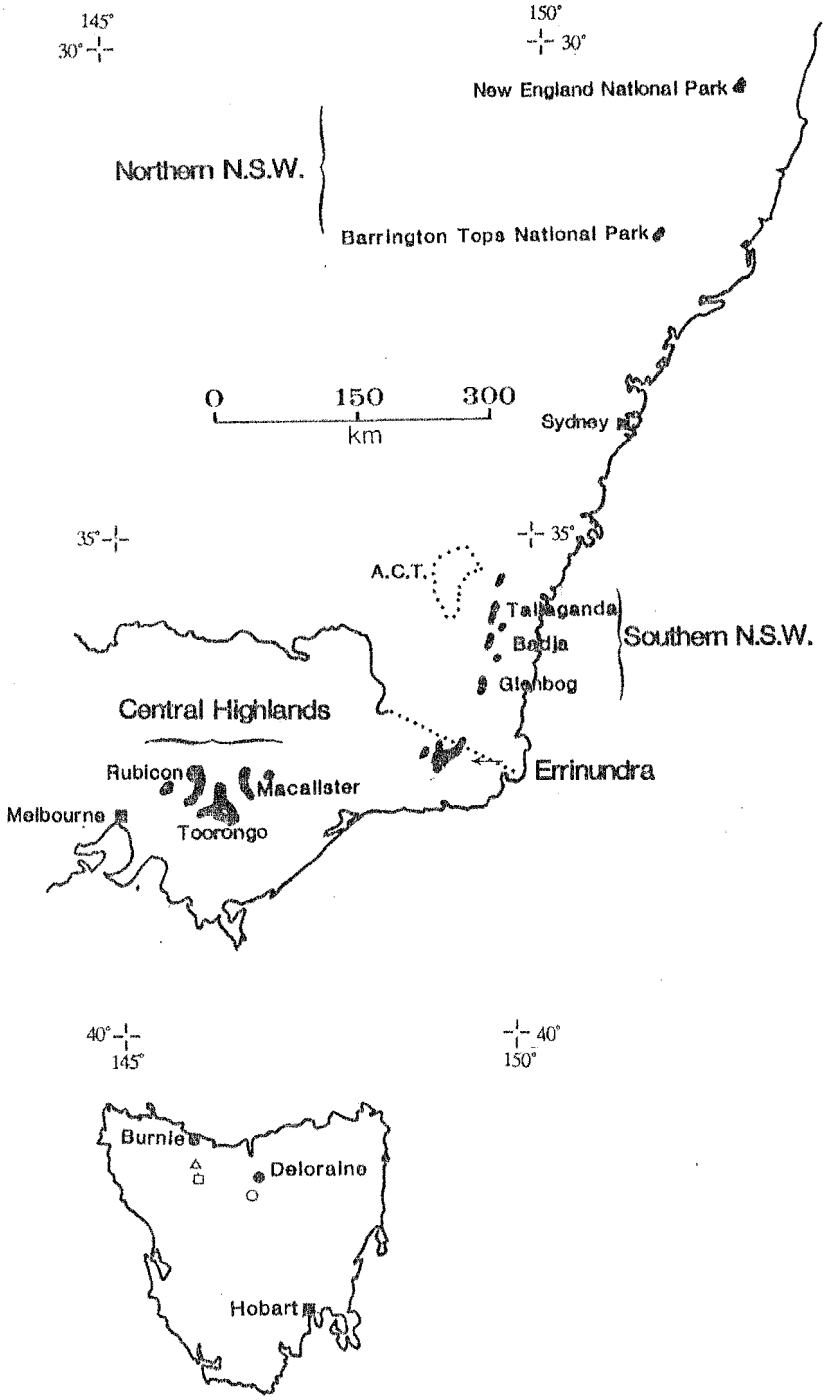


Figure 3.1 Natural distribution of *E.nitens* and the locations of three experimental sites in Tasmania, Hampshire (△), Racecourse Plains (□) and Huntsman Valley (○).

Table 3.1. Mean temperature and rainfall (mm $year^{-1}$) information for meteorological stations near stands of *E.nitens*. Source, Hall *et al.* (1981).

Station	Provenance	Temperature (°C)			Frosts	Rainfall
		Max.	Min.	Record low	(N° $year^{-1}$)	
Armidale	Northern N.S.W.	20	7	-10	50	795
Nimmitabel	Southern N.S.W.	16	4	-11	92	720
Bondi S.F.	Errinundra	17	2	-	-	1050
Bendoc	Errinundra	17	3	-8	-	815
Tanjil Bren	Toorongo	16	6	-6	13	1726

Table 3.2. Some differences in morphological characters of the two forms of *E.nitens*.

Character	"early-adult form"		"juvenile-persistent form"
	Errinundra[A]	Toorongo[B]	
Mean tree volume* (dm^3 at 5 years)	70	80	125
Trees with adult foliage* (% at 2 years)	85	67	12
Juvenile leaf size* (cm^2)	38	48	69
Trees with polyphenols** (%)	3	47	100
Bud shape***	rounded	rounded	angular
Pedicle length (mm)****	9.9	11.3	7.3
Valves****	exsert	exsert	insert

[A] Errinunda plateau and Goongerah localities.

[B] Mt.Toorongo and Tanjil Bren, and Christmas Creek (not volume and juvenile leaf size)

Source:-*, Pederick (1979); **, Pederick and Lennox (1979);***, this study (Figure 8.1); ****, this study (Table 8.4).

CHAPTER 4

DEVELOPING EXPERIMENTAL FROSTING METHODS

4.1 Introduction

Frost resistance is typically measured either in field trials subjected to natural frosts, e.g. Griffin *et al.* (1982b), or by artificially frosting whole plants or tissue samples, e.g. Rook *et al.* (1980) and Raymond *et al.* (1986), respectively. Field trials rely on the occurrence of frosts that will be harsh enough to discriminate between species, provenances and/or families, yet not kill all plants, which may include valuable genotypes. Such frosts may not occur when required. Controlled environmental facilities capable of producing freezing temperatures enable frost resistance studies whereby the type, severity and duration of frost conditions can be controlled. This permits research into physiological and genetic aspects of the variation in frost resistance in plants,with more accuracy and speed than can be achieved when relying on natural frost conditions.

Artificial frosting studies rely on two main criteria. Firstly, the ability of the controlled freezing apparatus to simulate natural frost conditions and secondly, the use of some method for assessing the effects of frost on the plant material. A number of fixed and portable freezing apparatus have been designed to simulate frosts under controlled conditions (see Table 4.1). Most of the large cabinets have been used exclusively for frosting whole plants, e.g. Aston and Paton (1973) and Robotham *et al.* (1978), whilst liquid cooling baths, similar to that used by Raymond *et al.* (1986), exclusively frost tissue samples and cannot handle whole plants. The freezing apparatus used in these studies (see 4.2.1 for a description) was capable of accurate temperature control and could handle both whole seedlings or tissue samples. Therefore, it offered the advantage of using either whole plants or tissue samples and investigating the nature of the relationship between damage to whole plants and tissue samples.

A number of direct and indirect methods of assessing frost damage to plant tissue have been used. The most simple, direct assessment merely scores the survival of either whole plants or tissue samples, e.g. Thomas and Barber (1974) and Sakai *et al.* (1981) respectively. More complex systems of damage evaluation have also been used which assess damage on the basis of proportional damage and/or recovery to various tissue types. Rook *et al.* (1980) used a visual assessment of the overall percentage damage to *E.regnans* seedlings,which placed each plant into one of six damage categories; a similar system to this was adopted for assessment of damage to field trials (see section 6.2).

Ashton (1958) developed a more elaborate system of visual estimation of leaf and

bud damage in *E.regnans* seedlings based on the average fraction of each leaf damaged (to the nearest quarter) and the proportion of total buds killed. Total damage was expressed as the mean of these two figures. A system similar to this was adopted for the evaluation of frost damage to whole seedlings, although stem height killed was used instead of proportionate bud death (because of differences in internode lengths of the provenances being compared) and leaf and stem damage were kept separate (see section 5.2). The suitability of these kind of leaf damage assessments depends upon both the accuracy and precision of visual assessment and the homogeneity of leaf size within a plant. In this study, leaves from the top four nodes were assessed, and 26% and 58% of all leaves inspected were undamaged or totally killed respectively, and hence subjective assessments were made on only 16% of leaves. Since *E.nitens* leaves from the top one or two leaf pairs assessed were usually not fully expanded and generally received more damage than lower leaves, mean leaf damage for the seedling overall may have been slightly overestimated. Where all leaves were undamaged or totally killed, the variation in leaf size would in no way bias mean leaf damage. However, any bias due to within-seedling variation in leaf areas should be similar across treatments (growth regimes, genotype etc.) since the pattern of leaf expansion for all seedlings was similar, i.e. only the first two leaf pairs were generally not fully expanded.

An indirect method of damage assessment was developed by Paton (1972) on frosted whole eucalypt seedlings using the percentage ratio of leaf dry weight to fresh weight. The underlying assumption of this method is that the relative amounts of frost killed and living tissue within the plant are reflected in the ratio. Although this method is quantitative and correlates well with actual survival (Paton 1972), it was not chosen since the removal of all leaves may affect seedling vigour and stem damage measurements.

Table 4.1 Various controlled freezing apparatus that have been used.

Frost type	Material*	Capacity (dm^3)	Cooling rates ($°C\ h^{-1}$)	Minimum temperature (°C)
Fixed apparatus				
radiation	seedlings	22550	6 to 240	-70 ± 0.3^a
advective	seedlings	2775	1 to 20	-20 ± 0.5^b
Portable apparatus				
advective	seedlings	625	2 to 10	-10 ± 0.5^c
advective	either	157	3 to 63	-26 ± 0.5^d
advective	tissue	24	?† to >5	-30 ± 0.1^e

*; whole seedlings, tissue samples or either

Source; a, Aston and Paton (1973); b, Robotham *et al.* (1978); c, Clare *et al.* (1984); d, this study; e, Raymond *et al.* (1986)

†, not stated

In addition, a number of indirect physiological tests have been used to quantify damage associated with freezing injury. These include, resistance to alternating electric current (Greenham and Daday 1957;Jaramillo 1984; Pelkonen 1984), electrolyte leakage (Dexter *et al.* 1932; Palta *et al.* 1977a,b), vital staining (Harwood 1981), protoplasmic streaming and plasmolysis (Li *et al.* 1979) and chlorophyll fluorescence analysis (Barnes and Wilson 1984). The objectivity of such tests is an obvious advantage, although their usefulness depends on the degree to which their results correlate with actual damage to the plant material. Most of these tests are usually carried out on tissue samples, viz., detached leaves or leaf discs/segments, and therefore have the added advantages that they can repeatedly and non-destructively sample plants of a wide range of sizes. Many of these tests have been used with eucalypts, e.g. resistance to alternating electric current and vital staining (Harwood 1981) and electrolyte leakage (Webb *et al.* 1983; Hallam 1986; Raymond *et al.* 1986). The determination of frost resistance in eucalypts using the electrolyte leakage method has been widely used in recent years and has been shown to be rapid, repeatable, discriminatory and non-destructive (Hallam 1986; Raymond *et al.* 1986). This method was adopted for many of the frost resistance studies reported in this thesis.

Examining various physiological and genetical aspects of frost resistance in *Eucalyptus* species have largely involved subjecting whole seedlings to controlled frosting treatments (Paton 1972, 1980, 1981; Harwood 1980; Rook *et al.* 1980; Menzies *et al.* 1981). Recent developments in the method of subjecting leaf disc samples to artificial frosting (Hallam 1986; Raymond *et al.* 1986) may mean that these methods may largely replace those involving frosting whole seedlings. The use of leaf discs for evaluating frost resistance in *Eucalyptus* species has a number of advantages over frosting whole seedlings, viz., it is more efficient in both time and space, more flexible since it can use material from plants of virtually any size and from various locations, allows results to be obtained more rapidly, and does not destroy plants which may be extremely valuable, e.g. artifically produced hybrids.

Although actual methodologies of subjecting whole seedlings and leaf discs to artificial frosting are reasonably well developed for eucalypts, it was considered necessary to assess a few critical factors of the imposed frost conditions on the development of damage, since the freezing apparatus was quite different to the radiation and advective frost rooms (Aston and Paton 1973; and Robotham *et al.* 1978, respectively) and the liquid-cooled bath (Raymond *et al.* 1986), where so many of the previous studies have been conducted.

4.2 Materials and Methods

4.2.1 The freezing apparatus used

Artificial frosting was conducted in an air-filled freezing chamber painted black inside, with internal dimensions of 75 cm height and 50 cm width and depth. The top 12 cm of the internal space was occupied by a variable-speed air-circulation fan located above an evaporator and refrigerant coils (effective carrying capacity of the chamber was therefore

0.1575 m^3).

Cooling of material within the chamber took place by the principle of convective heat loss. Refrigerant was directed either through the evaporator in the chamber (when cooling was necessary) or else through an external evaporator (when cooling was not necessary) by means of two solenoid valves. Temperature was initially sensed by an integrated circuit device (AD 590LH). However, early in frosting studies this was replaced by a minature transistor (type MMT 2369) which operated alternatively at two discrete collector currents. The transistor gave a much lower temperature rise in still air, due to internal dissipation, than the integrated circuit, i.e. 0.76 c.f. 0.014°C. The output from either sensor was fed into an electronic circuit which controlled operation of the solenoid valves necessary to achieve the required rate of cooling (between 0.05 and 1.05°C min^{-1}) and desired temperature (to -26 ± 0.1°C, i.e. the ambient temperature monitored by the sensor). Throughout all artificial frostings output from the temperature sensing device was recorded on a chart.

4.2.2 Growth treatments

Seed was germinated (on moist vermiculite in plastic punnets) in a heated glasshouse (*c.* 25°C day/15°C night) and transplanted into polythene pots (6 cm diameter, 15 cm high), one seedling per pot. A steam sterilised potting medium was used comprising a sandy loam/peat/sand mix in the ratio 5:3:1. The nutrient status of the potting mix was supplemented with Osmocote (Sierra Chemical Europe B.V.; 18%N, 4.8%P, 8.3%K), dolomite, and blood and bone at the rates of 0.5, 1.0 and 0.6 g $seedling^{-1}$ respectively. Seedlings were grown in a heated glasshouse (25°C/15°C) and when *c.* 25 to 45 cm tall were selected for various experiments.

During the winter months, the seedlings were sometimes infected with *Botrytis* spp. which necessitated regular spraying with various fungal sprays, e.g. Benlate {Du Pont (Australia) Ltd.}, and a reduction in the watering regime.

4.2.3 Developing frosting techniques for whole seedlings

Seedlings were transferred to a growth cabinet {16°C day (10 h) and 12°C night} and after 11 days were selected for three basic experiments, designed to assess critical factors in the method of artificial frosting being used. Although these and other factors had already been tested with eucalypts by Harwood (1981), it was considered necessary to check the applicability to frosting studies with *E.nitens*. Three experiments were conducted (Table 4.2). The underlying objective of these experiments was to determine a frosting regime (rate of cooling, duration of frost etc.) which simulated natural frost conditions, capable of resulting in assessable levels of damage, yet was efficient in terms of time and material resources. Unless otherwise stated, the following procedures were used.

Seedlings were placed in a white plastic box (45 cm long, 35 cm wide and 10 cm tall) and the roots and pots effectively insulated with plywood placed around the four sides of the box and a 3 cm layer of dry vermiculite covering the soil surface of the pots. The box of

seedlings was then placed in the freezing chamber and the temperature lowered at a rate of *c.* 0.8°C min^{-1} to 2.0°C, and thereafter, lowered at a rate of 0.2°C min^{-1} to the desired frost temperature.

After 90 min at the frost temperature, the chamber was allowed to warm to -1.0°C (usually taking 30 min). Seedlings were then quickly transferred to a cabinet at the same photoperiod and temperature regime as the growth treatment before frosting. After seven days, seedlings were returned to the glasshouse where they were assessed for frost damage after an additional seven days, i.e., 14 days after frosting. The percentage leaf surface area damaged, for each leaf of the top four leaf pairs on each seedling, was visually assessed (to the nearest 5% where possible). In all cases the top leaf pair was not fully expanded whilst the two lowest leaf pairs were always fully expanded and healthy. Leaf pairs below these were not assessed since they were sometimes absent or too close to the radiating surface of the vermiculite. Seedling mean leaf damage was calculated as the mean of the percentage damage scores from all leaves.

1. Spraying seedlings with water before frosting

The purpose of this experiment was to determine whether the extent of damage to whole seedlings was affected by the presence of water on plant tissue at the time of frosting, since frosts often occur with a dew which leads to the freezing of the water that has settled on the surfaces of plant tissues. Twelve seedlings were selected for each of three planned frostings, viz., -3.0, -3.5 and -4.5°C (Table 4.2). Half of the seedlings were carefully wetted, each with a single fine mist spray of distilled water, whilst the other half of the seedlings were shielded from the spray.

2. Rate of cooling to frost temperature

The purpose of this experiment was to determine whether the extent of damage to whole seedlings was affected by the rate at which seedlings were cooled to a frost temperature. Under natural conditions in Australia, during the months when frosts are likely to occur, the ambient temperature is usually lowered at a maximum rate of 6°C h^{-1} (Davidson and Reid 1985). If faster rates of cooling could be used without dramatically altering the resultant damage this would allow more frosts to be conducted in a given time, particularly when minimum temperatures of increasing severity (more negative) are used. Groups of 12 seedlings were cooled to the frost temperature of -3.8°C at one of three different rates of cooling from 2.0°C (standard cooling at 0.8°C min^{-1} to 2.0°C), viz.,(1) 0.05°C min^{-1} (3.0°C h^{-1}); (2) 0.2°C min^{-1} (12.0°C h^{-1}); and, (3) 0.8°C min^{-1} (48.0°C h^{-1}). Each group consisted of six seedlings grown under controlled conditions {11 days at 16°C day (10 h)/12°C night} and a further six seedlings which had been continually grown in the glasshouse (Table 4.4). Otherwise standard conditions applied.

Table 4.2 Details of preliminary frosting experiments.

Experiment	Nº treatments	Frost temperatures (°C)	Nº samples
Frosting whole seedlings			
Spraying seedlings with water	2 (wet, dry)	-3.0, -3.5, -4.5	6
Rate of cooling	3 (3, 12 or 48°C h^{-1})	-3.8	12
Duration of frost	3 (45, 90 or 180 min)	-3.3	10 or 12
Frosting leaf discs			
Time of reading conductivity	2 (shaken, unshaken)	-13 and unfrosted	10
Evaluating maximum conductivity	4 (heating or freezing)	-8.0, -13.0	8 or 10
Floating leaf discs on either surface	2 (abaxial, adaxial)	-4.5, -13.0	10
Size and number of discs	6 (see Table 4.3)	-3.7, -5.0, -6.3	8
Post frost conditions	3 (thawing rates)	-5.6, -6.8, -8.0	10
Nodal variation	6 (nodes)	-3.8, -5.0, -6.2	4
	5 (nodes)	-4.7, -5.4, -6.8	4
Rate of cooling	3 (3, 12 or 48°C h^{-1})	-4.7, -6.4, -7.5	12

Table 4.3 Size and numbers of leaf discs used to assess relative frost resistance of *E.nitens* seedlings. Frost temperatures corresponding to relative conductivity readings of 50% (T50) are indicated; those with similar letters are not significantly different (P<0.01).

Particulars	Diameter of discs(mm)					
	6.0	6.0	6.0	8.5	12.0	14.5
No. discs	2	4	6	1	1	1
Area (mm^2)	56	112	168	56	112	168
Cut tissue (mm)†	38	76	114	27	38	46
Area/cut tissue (mm^2 mm^{-1})	1.5	1.5	1.5	2.1	2.9	3.6
T50 (°C)	-4.4a	-4.5ab	-4.7abc	-4.4a	-4.8bc	-5.0c

†, disc circumference x number of discs

3. Duration of frost

Field frosts can occur for varying lengths of time (see Figure 7.1). The purpose of this experiment was to determine a suitable duration at the selected frost temperature. Ten or 12 seedlings were chosen for exposure to an artificial frost of -3.3°C for one of three durations, viz., (1) 45 min; (2) 90 min; and, (3) 120 min (Table 4.2).

4.2.4 Developing experimental techniques for leaf discs

The dimensional limitations of the freezing chamber, coupled with the advantages of using leaf discs rather than whole seedlings and the demand from other users for the chamber, necessitated investigations into using leaf disc samples for determining frost resistance. For instance, a maximum of *c.* 110 vials containing leaf discs could be practically and efficiently handled in the chamber, whereas an absolute maximum of 30 whole seedlings (in the sized pots used) could be frosted in the chamber. In general, frosting whole seedlings rather than tissue samples to evaluate frost resistance required 20 times the number of seedlings and at least twice the number of frostings when using this apparatus.

Seven small scale experiments were conducted to check the validity of the leaf disc method of frosting with *E.nitens* (Table 4.2). A method similar to that of Hallam (1986) was chosen for these experiments, since Hallam developed her technique on the same freezing chamber. Unless otherwise stated, the following procedures were used;

a) discs (14.5 mm diameter) were carefully cut with a cork borer

b) they were immediately placed in a glass vial (2.5 cm diameter, 5.0 cm high)

c) each vial had 0.2 ml distilled water and 0.1mg AgI added {this facilitated freezing; see Hallam (1986)}

d) vials were stood on a wire base in the freezing chamber

e) the temperature of the chamber was lowered to 2.0°C at the rate of 1.0°C min^{-1} and thereafter at 0.2°C min^{-1} to the frost temperature

f) the frost temperature was maintained for 100 to 110 min

g) the chamber was turned off and the vials removed

h) after 30 min at room temperature 8.0 ml distilled water was added to the vials as a bathing medium and a plastic cap placed on each vial

i) 20 to 24 h later the conductivity of the bathing medium (CI) was read to the nearest µS cm^{-1} with a pp1042 probe attached to a Radiometer Microvoltmeter

j) the vials were heated in a microwave oven until the bathing medium was at *c.* 70°C

k) the total conductivity (CT) was read 24 h later

l) relative conductivity (CR) was calculated as, CR = CI/CT

CR was plotted against frost temperature and relative frost resistance evaluated as the temperature resulting in a relative electrical conductivity reading of 50% (T50).

1. Time of reading conductivity

The purpose of this experiment to determine at what time conductivity readings should be made, since the time at which conductivity readings are made needs to be standardized so that comparable assessments of conductivity are made. The work of Palta *et al.* (1977a) and Hallam (1986) indicates that the conductivity of the bathing medium (for a given level of damage) rises to a maximum value after a length of time. In some species suffering low levels of damage the conductivity may subsequently fall due to post-frost recovery (Palta *et al.* 1977b).

Conductivity readings were made at various times (to 28 h) after addition of the bathing medium to four treatments containing duplicate discs from each of five *E.nitens* seedlings. The treatments were unfrosted or frosted to -13°C, each of which was incubated on or off a shaker. Samples were kept at room temperature (*c.* 20°C).

2. Method of evaluating maximum conductivity

The purpose of this experiment was to determine an efficient method of evaluating total conductivity for use in calculating relative frost damage. The work of Eldridge *et al.* (1983) showed that comparative frost resistance studies with *Eucalyptus* species using the conductivity method requires standardization of conductivity readings to allow for variations in ionic levels amongst tissue from different leaves. The usual standardization procedure is to measure the total conductivity (CT) of the tissue and to incorporate the initial conductivity following the frost (CI) with the total conductivity into some index of relative conductivity (CR). For example, Raymond *et al.* (1986) and Hallam (1986) use the following indices respectively;-

$CR = ((CT-CI)/CT)^{0.5}$

$CR\% = (CI \times 100)/CT$

CT was evaluated 24 h after single discs from *E.nitens* seedlings had received one of the following four treatments:-

a) frosted to -8.0 or -13.0°C,

b) immersed in liquid nitrogen for 30 or 60 sec,

c) heated in a microwave oven so that the bathing medium reached 55 or 70°C, and

d) slowly heated in a water bath so that the bathing medium reached 70°C.

Discs in treatments (a) and (b) had 8.0 ml distilled water added 30 min after treatment, whilst those in treatments (c) and (d) had distilled water added before treatment. No vials had Ag I added. The conductivity of the added bathing medium (assumed CT) was determined 24 h after its addition to the vials.

3. Floating discs on abaxial or adaxial surface

The purpose of this experiment was to determine if leakage of cellular electrolytes into the bathing medium was affected by which surface of the leaf disc was in contact with

the bathing medium. Duplicate discs were taken from the same 10 seedlings as above, frosted to -13.0°C and the discs floated with abaxial or adaxial surface in contact with the bathing medium (10 ml). This procedure was repeated with a new series of discs frosted to -4.5°C. The conductivity of the bathing medium was determined 24 h later.

4. Size and number of leaf discs

The purpose of this experiment was to assess the effects of the dimensions and amount of leaf tissue sampled on frost resistance estimates. Hallam (1986) used six discs, each 6 mm in diameter, whilst Raymond *et al.* (1986) used single discs of 8 mm diameter. Effects of disc size and the total surface area sampled on conductivity readings were determined by frosting duplicate samples from four seedlings {hardening at 25°C day(8h)/3°C night (16h)} to one of three temperatures -3.7, -5.0 or -6.3°C using standard procedures. Leaf tissue was sampled according to six treatments (Table 4.3). Discs of three surface area categories were selected, i.e. 56, 112 and 168 mm^2. Within each of these, the surface area was obtained from either two or more 6 mm discs, or a single disc of larger diameter. All tissue sampled within a replication on a seedling , i.e.15 discs per frost, came from the same fully expanded healthy leaf.

5. Post frost conditions

The purpose of this experiment was to assess the effects on relative conductivity readings of the time at which the bathing medium was added after frosting. Six discs from each of 10 seedlings were frosted to temperatures of -8.0,-6.8 or -5.6°C. At the completion of the frost period two of the samples from each seedling had 8.0 ml distilled water added immediately and were kept at room temperature (*c.* 20°C), two of the remaining samples were quickly sealed in their vials (with plastic caps) and stored at 2.0°C, and the remaining two samples were left to stand at room temperature for 40 min before the addition of 8.0 ml of distilled water. The following day all 60 vials stored at 2.0°C were allowed to equilibriate with room temperature before adding 8.0 ml distilled water. The samples were in the 2.0°C conditions for between 15 and 20 h. Conductivity readings from all samples were made 24 h after the addition of the bathing medium (CI) and 24 h after microwave heating (CT).

6. Nodal variation

Variation in frost resistance amongst leaves from different nodes on a plant was examined on two separate occasions by frosting discs from leaves at various nodes on four seedlings. Standard preparation, frosting, post frost and conductivity procedures applied. Firstly, seedlings from two open-pollinated families from the Toorongo provenance that were hardening outdoors (see Section 6.2) were sampled by cutting two discs from leaf pairs at each of six consecutive nodes from the fifth node (cotyledons as node 0). On all seedlings, leaves below the fifth node had fallen whilst leaves at node 10 were not fully

expanded. However, node 10 was the highest node at which there was sufficient leaf material to cut the six discs required for frosting to -3.8, -5.0 and -6.2°C. Secondly, samples were taken from seedlings of two open-pollinated Northern N.S.W. families that were being artificially hardened {25°C day (8 h)/3°C night (16 h)}. In these seedlings leaves from only five nodes were sampled, i.e. generally nodes 5,9,11,15, and 17. Actual nodes sampled varied slightly because of a marked reduction in leaf area of the first few leaf pairs that expanded after imposition of the hardening regime. However, leaf shape was unaffected (see also Shepherd *et al.* 1976).

7. Rate of cooling

The effects of three rates of cooling on estimates of frost resistance were examined using single discs from each of two leaves on six seedlings from an open-pollinated Northern N.S.W. family (artificially hardened as above). All samples were cooled to 2.0°C at a rate of 0.8°C min^{-1} (48°C h^{-1}) and thereafter cooled at either the standard rate of 0.2°C min^{-1} (12°C h^{-1}), or the minimum rate of 0.05°C min^{-1} (3°C h^{-1}), or left at 0.8°C min^{-1}. Frost temperatures of -6.4 and -4.7°C were used at all three cooling rates and also -7.5°C with the slowest cooling rate. Standard post-frost procedures applied.

4.2.5 Statistical analysis

Percentage leaf damage scores were not normally distributed and analysis was undertaken using non-parametric tests. The scores were arc-sin transformed and Kruskal-Wallis one-way analysis of variance used to test amongst treatments and one-tailed Mann-Whitney U-tests were used to test differences between pairs of treatments (Anon. 1986). Frost temperatures causing 50% loss of cellular electrolytes (T50) were approximately normally distributed and analysis amongst and within treatments were carried out using analysis of variance.

4.3 Results

4.3.1 Temperature variation within the freezing apparatus

When whole seedlings were frosted, the temperature sensor of the freezing chamber itself was positioned at the level of the second highest leaf pair. The actual ambient temperature, once the desired temperature was reached, generally varied by 0.3°C or less in one horizontal plane and increased by 0.2°C from the top leaf pair to the lowest leaf pair assessed (*c.* 12 cm distance). Soil temperatures were never lower than 3.0°C and were generally warmer than 4.0°C. Hence, the frost temperature selected represents an average temperature to which the seedlings were exposed with the lowest few centimetres of the stem and roots being the only portions of the seedlings subjected to temperatures differing by more than +0.3°C.

The effects of vertical stratification of air temperature within the freezing apparatus were avoided when using leaf discs since all samples were in one horizontal plane. Where 84 vials or less were used, the variation in sample temperature was generally less than 0.4°C at steady state (measured by copper-constantan thermocouples placed in vials next to leaf discs). Where up to 108 vials were used, the absolute variation in temperatures of leaf disc samples did not exceed 0.8°C and averaged 0.3°C, for frost temperatures from -3.0 to -12.8°C. The effects of temperature variation (> 0.3°C) throughout the freezing chamber on comparability of estimates of relative frost resistance were minimised by stratifying sections of the chamber according to mean temperature at steady state (based on thermocouple recordings), assigning equal replications of each treatment to the strata and using the mean temperature of each strata in calculation of relative frost resistance parameters, e.g. T50. Samples which remained at the lowest temperature were located under the section of the cooling coil where the refrigerant entered, whilst those which remained at the warmest temperature were located under the section of the coil from which the refrigerant left. The positioning of the temperature sensor at the top of the vials (2.5 cm above discs) coupled with some vertical stratification in temperatures would probably explain why the temperatures of samples were always *c.* 1.0°C warmer than chamber temperature (Figure 4.1).

Freezing exotherms indicate that samples generally froze at temperatures between -2 and -4°C (Figure 4.1). Where the frost temperature was in this range, some samples did not freeze until near the end of the frost duration and others did not freeze at all, though generally 95% of samples had frozen by the end of the frost duration. Without AgI, leaf samples may remain unfrozen at -6°C (Hallam 1986).

4.3.2 Effects of frost conditions on damage to whole seedlings

Mean leaf damage scores for whole seedlings frosted in the three preliminary experiments are shown in Table 4.4. The seedlings were relatively unhardened, with 50% leaf damage occurring at temperatures of about -3.5°C.

1. Spraying seedlings with water before frosting

There were no significant differences (P>0.05) in mean leaf damage scores, at any of the three frost temperatures, between the seedlings kept dry or those sprayed with water before frosting. Leaves from different seedlings were often touching but little water was transferred from seedlings that had been sprayed to those that were not sprayed. Data from separate studies of thermocouples placed against adaxial surfaces of leaves reveal that freezing exotherms occur concurrently for different leaves within one seedling. Although ice nucleation is rapidly transferred through the tissue of one seedling, there were no data which indicated that initiation of ice nucleation in any one seedling may confer subsequent ice nucleation in adjoining seedlings. The fact that seedlings can freeze in the natural environment with low humidities, i.e.black frosts, indicates that the presence of surface

water on seedlings *per se* may not influence the level of damage.

2. Rate of cooling to frost temperature

The more rapid the cooling rate to a frost temperature of -3.8°C the greater the mean leaf damage that was sustained in *E.nitens* (Table 4.4). However, only the slowest rate (0.05°C min^{-1}) and the fastest rate (0.8°C min^{-1}) were significantly different overall ($P<0.05$). The cooling rate of 48°C h^{-1} resulted in an initial and temporary (*c.* 5 min) "overshoot" of the programmed frost temperature by *c.* 0.6°C. Since the range in frost temperature over which damage passed from slight (*c.* 0%) to severe (*c.* 50%+) was less than 1.0°C (Table 4.4), this overshoot *per se* , rather than the rate of cooling, could be at least partially responsible for increased damage between the 0.8°C min^{-1} and slower cooling rates.

Seedlings grown in the glasshouse (25°C/15°C) on average suffered equivalent levels of damage compared with seedlings conditioned in the cabinet (16°C/12°C), except for frost temperatures reached by cooling at 0.2°C min^{-1}. However, the data are based on only six replications and a single frost temperature, which in all cases caused a high mean level of damage, i.e. above 50%. Paton (1972) found that *E.viminalis* seedlings grown at 10°C night temperatures suffered less damage than seedlings grown in a glasshouse.

3. Duration of frost temperature

Increasing the duration of -3.3°C frost resulted in greater mean leaf damage to *E.nitens* seedlings (Table 4.4). However, none of these differences were significant ($P>0.05$). In all experiments there was a large amount of variation in the damage score amongst individual seedlings at a given temperature. Often within a single treatment some seedlings would be killed whilst others may be undamaged. Increasing the number of replications within a treatment may have increased the significance of some of the differences reported. However, in so doing this may necessitate multiple runs of the same frost temperature because of the physical limitations of the freezing chamber.

Table 4.4 Mean (± S.E.) leaf damage (%) for whole *E.nitens* seedlings grown and frosted under various treatments in preliminary frosting experiments. Scores with similar letters, within experiments, are not significantly different (P>0.05)

Experiment	Treatment	Frost (°C)	No. seedlings	Leaf damage (%)
1, surface water	leaves wet	-3.0	6	7± 4.6 a,
	leaves not wet	-3.0	6	0± 0.2 a
	leaves wet	-3.5	6	44±20.0 b
	leaves not wet	-3.5	6	48±15.0 b
	leaves wet	-4.5	6	86±14.0 c
	leaves not wet	-4.5	6	98± 1.7 c
2, rate of cooling*	0.05°C min^{-1}			
	(1) 25°C/15°C**	-3.8	6	53±18.9 a
	(2) 16°C/12°C	-3.8	6	59±15.2 a
	Mean	-3.8	12	56±11.8 a
	0.2°C min^{-1}			
	(1) 25°C/15°C	-3.8	6	92±5.3 b
	(2) 16°C/12°C	-3.8	6	61± 14.3 a
	Mean	-3.8	12	76± 8.6 ab
	0.8°C min^{-1}			
	(1) 25°C/15°C	-3.8	6	92± 5.0 b
	(2) 16°C/12°C	-3.8	6	93± 3.6 b
	Mean	-3.8	12	93± 2.9 b
3, frost duration	45 min	-3.3	10	38±12.2 a
	90 min	-3.3	10	60±11.9 a
	180 min	-3.3	12	61±16.2 a

*, cooled to 2.0°C at 0.8°C min-1

**, glasshouse (25°C/15°C) or growth cabinet (16°C/12°C) grown plants

4.3.2 Experimental techniques with leaf discs

1. Time of reading conductivity

The relationship between conductivity and time of incubation is shown in Figure 4.2. The conductivity readings from unfrosted discs significantly increased ($P<0.001$) with time to relatively stable readings by 20 h but were not affected by shaking ($P>0.05$). The conductivity readings from what was effectively frost killed discs (-13°C) significantly increased with time and shaking ($P<0.001$). Maximum readings were not achieved until after 24 h. Although conductivity readings from shaken samples were higher than those from unshaken samples, the difference was only of the order of 2 to 3 $\mu S\ cm^{-1}$ in the time period 20 to 28 h,during which time near maximum readings were obtained. Hence, at the range of extremes from relatively little (unfrosted) to full ionic leakage (-13°C), maximum conductivities are achieved at about 24 to 28 h after addition of the bathing medium, and at this time the effects of shaking are only slight.

2. Method of evaluating total conductivity

Generally speaking, all four methods of evaluating total conductivity produced high conductivity readings. Immersion in liquid nitrogen (for 30 sec) was the only method with a significantly ($P<0.05$) different, and lower result (Table 4.5). Hence, freezing the samples to temperatures about 5°C below critical temperatures (viz. T50) or in liquid nitrogen (60 sec), or heating the bathing medium to between 55 and 70°C should give similar estimates of total conductivity. Using a microwave oven to heat discs provided the quickest method which gives comparable values to that of total frost kill (Table 4.5).

3. Floating discs on abaxial or adaxial surface

There were no significant differences ($P>0.05$) between the mean conductivities (CI) obtained from floating discs on either the abaxial or adaxial leaf surface (Table 4.5). This applied for both severely damaged discs (-13°C) and partially damaged discs (-4.5°C). However, in both instances the trend was for slightly higher conductivities from discs that were floated on their adaxial surface. The adaxial surface of juvenile eucalypt leaves has a greater stomatal density than the abaxial surface and this may explain the slight difference between the two mean conductivities. However, leakage from discs would appear to be largely independent of the leaf surface in contact with the bathing medium.

4. Size and number of leaf discs

Relative conductivities increased with both frost temperature and decreasing area of tissue sampled (Figure 4.3). Single discs gave consistently lower relative conductivities than

6 mm discs of comparable sample area. Consequently, the mean temperatures at which 50% leakage of cellular electrolytes occurs (T50) was significantly ($P<0.05$) affected by the size of the discs, i.e. 6 mm or larger, and the surface area of leaf tissue ($P<0.01$). Single larger discs gave lower (more negative) T50 values than 6 mm discs of comparable area, except for the lowest surface area of 56 mm^2. Two, four or six discs, of 6 mm diameter, gave mean T50 values that were not significantly different from each other ($P>0.05$), whereas increasing surface area of single discs resulted in increasing frost resistance estimates (lower T50). Single discs offer the advantage over multiple discs in that they are more rapidly processed and leave the remaining leaf tissue with relatively lower amounts of exposed, cut edge (Table 4.3).

5. Post frost conditions

For both families of *E.nitens* , there were no significant differences at each of three frost temperatures between mean relative conductivity values from discs that were placed at 2.0°C for 15 to 20 h before adding the bathing medium and discs that were allowed to thaw at room temperature for 40 min before adding the bathing medium (Figure 4.5). However, adding the water immediately after the frost, whilst samples were frozen and causing a rapid thaw, resulted in the highest relative conductivities at all frosts, and resulted in T50 estimates that were between 1.5 and 1.8°C warmer than those of the more slowly thawed samples ($P<0.001$). The family from the Toorongo provenance was significantly ($P<0.01$) less frost resistant than family from the Rubicon provenance.

6. Nodal variation

There were significant differences in relative frost resistance ($P<0.01$) between nodes and seedlings for seedlings hardening outdoors (Figure 4.6a), but only seedling differences were significant ($P<0.05$) for seedlings being artificially hardened (Figure 4.6b). However, in both cases the trend was for upper nodes (often not fully expanded) and lower nodes (often chlorotic) to be characterised by higher relative conductivities, and hence lower frost resistance than fully expanded, healthy leaves. Hence, recently expanded, mature, healthy leaves should be choosen as this is likely to reduce a source of variation.

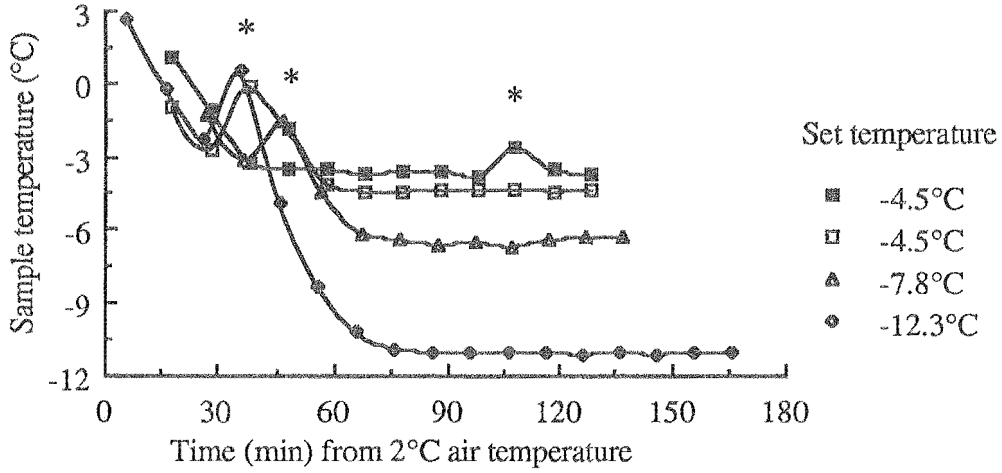


Figure 4.1 Changes in temperatures of leaf discs during frosting to set temperatures of -4.5, -7.8 and -12.3°C. Freezing exotherms are indicated (*).

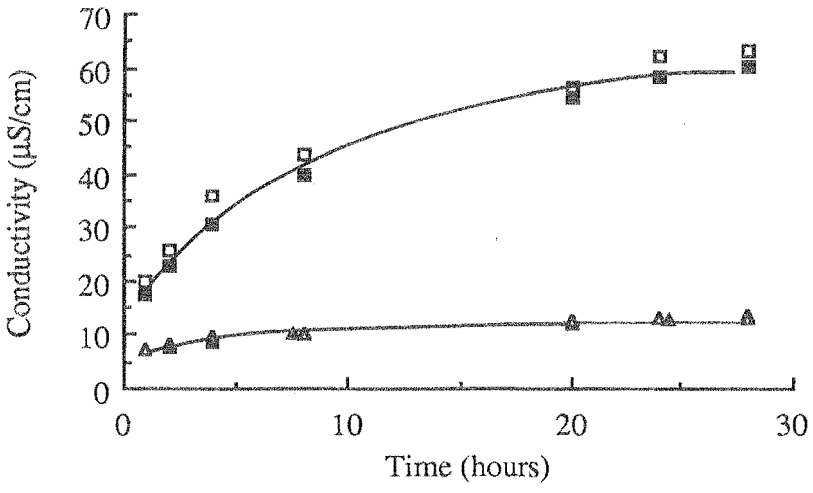


Figure 4.2 Relationships between mean electrical conductivity of the bathing medium containing leaf discs and time. Leaf discs were frosted to -13°C, and incubated on (□) or off (■) a shaker, or unfrosted and incubated on (△) or off (▲) a shaker.

Table 4.5 Mean (± S.E.) electrical conductivities ($\mu S\ cm^{-1}$) of the bathing medium containing leaf discs of *E.nitens* seedlings 24 h after (a) treatment by one of four methods aimed at inducing total cellular electrolyte leakage and (b) floating discs abaxial or adaxial surface following a -13 or -4.5°C frost. Also shown are estimates of the number of vials that could be treated h^{-1}.

Method	Conductivity	Vials treated h^{-1}	No. discs
(a)			
First group of seedlings			
-13°C	62± 3.7	100	8
liquid nitrogen (60 sec)	61± 3.0	50	8
Microwave oven (70°C)	60± 2.8	300	8
Second group of seedlings			
-8°C	67± 3.7	100	10
liquid nitrogen (30 sec)	61± 2.8	100	10
Microwave oven (55°C)	71± 5.2	300	8
Microwave oven (70°C)	76± 6.5	300	8
Water bath (70°C)	72± 5.2	200	8
(b)			
-13°C on abaxial	48± 2.6	-	10
-13°C on adaxial	52± 2.2	-	10
-4.5°C on abaxial	13± 1.0	-	10
-4.5°C on adaxial	15± 1.9	-	10

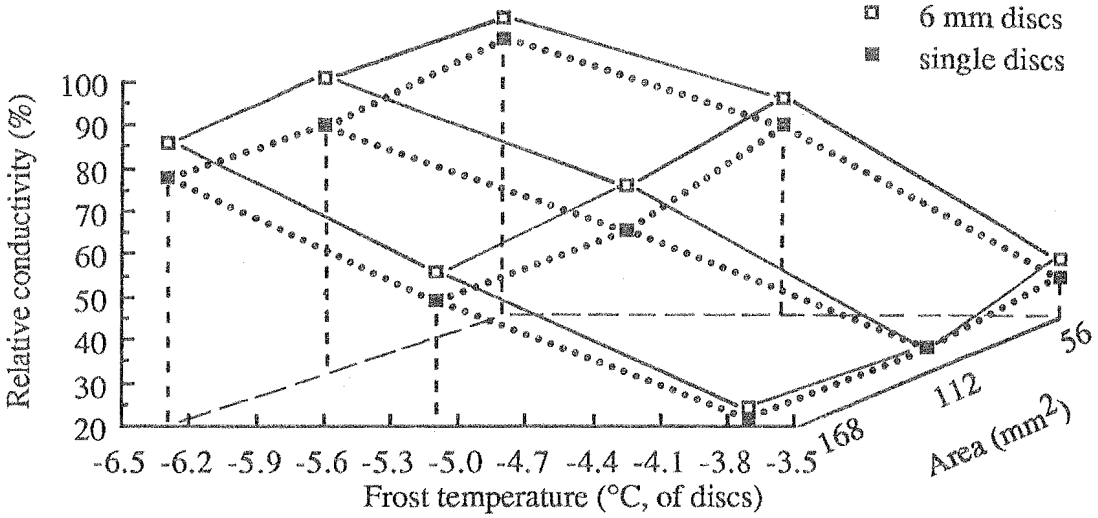


Figure 4.3 Relationships between mean relative electrical conductivity of the bathing medium containing leaf discs and frost temperature, for leaf discs of three tissue areas, from discs of 6 mm diameter or a single larger disc.

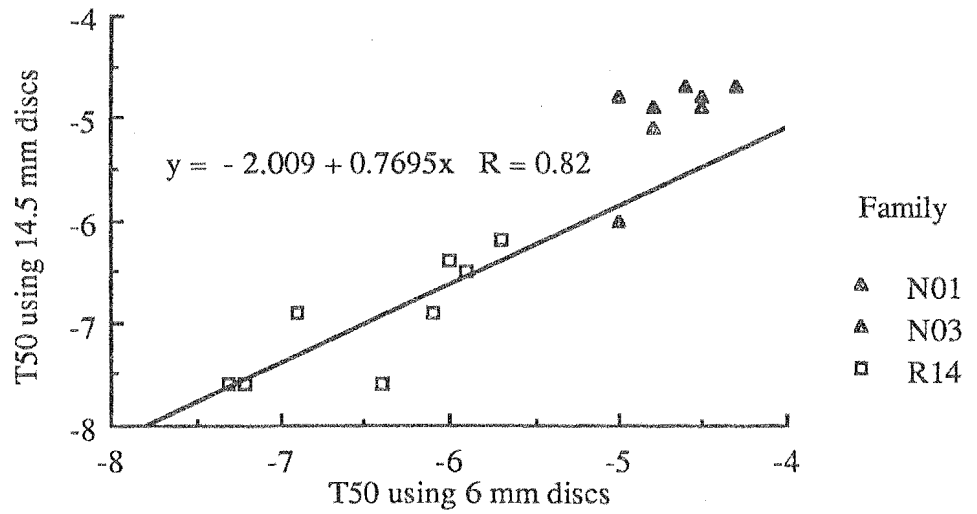


Figure 4.4 The relationships between relative frost resistance estimates (frost temperature at which 50% leakage of cellular electrolytes occurs) derived using a single 14.5 mm disc or six discs of 6 mm diameter. Leaf discs were sampled from potted seedlings (N01 and N03) and three-year-old plantation trees (R14). The linear regression for the data is shown.

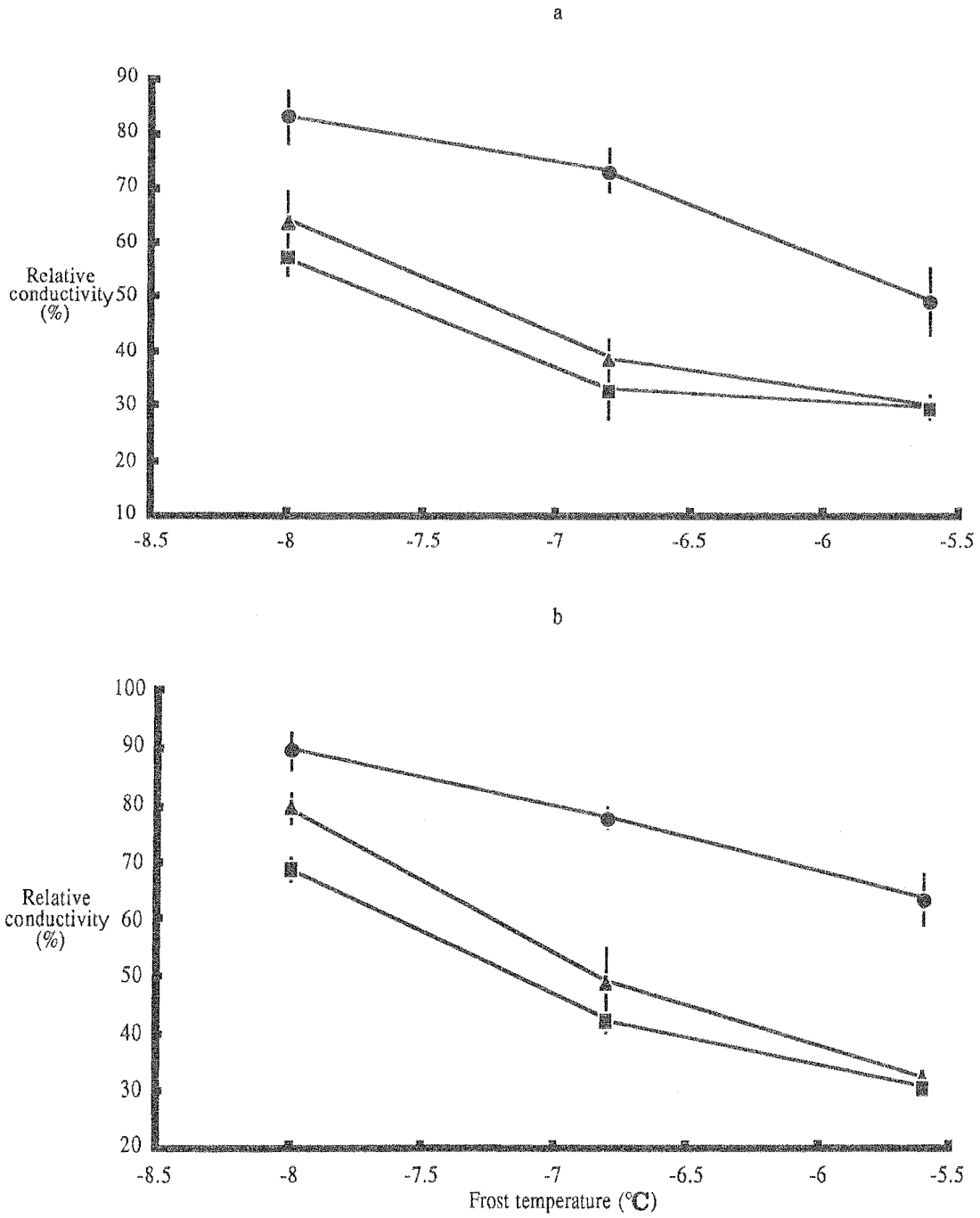


Figure 4.5 Relationships between relative electrical conductivity of the bathing medium and frost temperature, for leaf discs of *E.nitens* seedlings from the (a) Toorongo and (b) Rubicon provenance. Post-frost conditions are (●) water added immediately after frosting, (▲) water added 40 min after frosting, and (■) water added 15 to 20 h after post-frost storage at 2°C. Vertical bars are ± S.E. (≥ 2%).

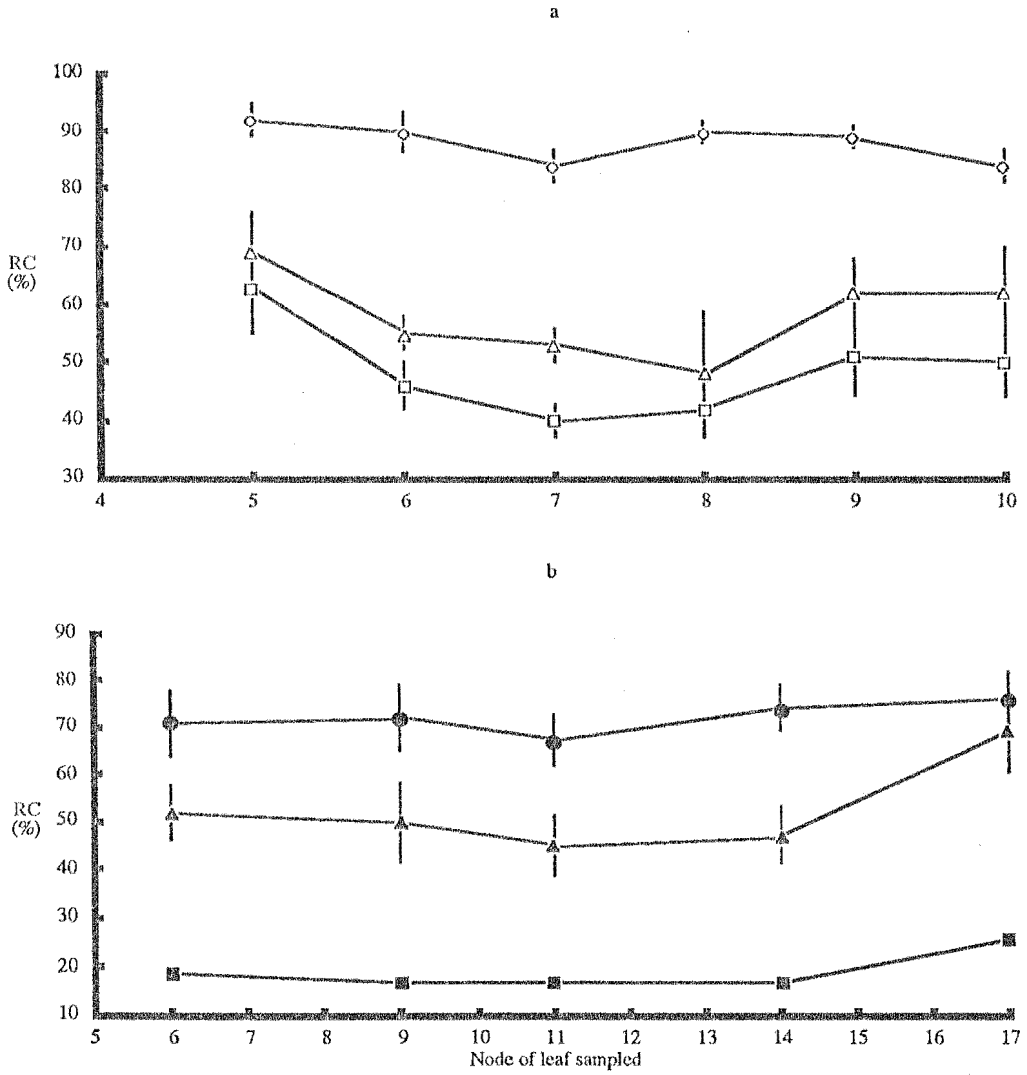


Figure 4.6 Relationships between relative electrical conductivity of the bathing medium containing frosted leaf discs (RC) and node of leaf sampled for *E.nitens* seedlings from the (a) Toorongo and (b) Northern N.S.W. provenance. Leaf discs were subjected to frost temperatures of -3.8 (□), -5.0 (△), -6.2 (○), -4.7 (■), -5.4 (▲) or -6.8°C (●). Vertical bars are ± S.E. (≥ 2%).

7. Rate of cooling to the frost temperature

There were no significant differences in the mean relative electrical conductivities of the bathing media containing leaf discs subjected to three rates of cooling to -4.7°C (sample temperature), but at the more severe frost of -6.4°C mean relative electrical conductivities of discs cooled at either 0.2 or 0.8°C min^{-1} were approximately twice that of discs cooled at 0.05°C min^{-1} (Table 4.6). Consequently, mean T50 values for *E.nitens* leaf discs cooled to frost temperatures at 0.05°C min^{-1} (3°C/h) were *c.* 1.5°C lower than those cooled at 0.2 and 0.8°C min^{-1}, viz. -7.0, -5.6 and -5.4°C respectively ($P<0.01$). In contrast to the rate of cooling experiments with whole seedlings, these seedlings were reasonably well hardened, and the programmed frost temperatures with the 0.8°C min^{-1} rate of cooling were initially set at 0.5°C warmer than that intended and adjusted, when these were reached, down to the desired temperatures. This avoided any "overshoot" of the intended frost temperature which occurred when whole seedlings were frosted.

Table 4.6 Mean (± S.E., n=12) relative electrical conductivities (%) for leaf discs from hardened *E.nitens* seedlings cooled to various frost temperatures (°C) at three cooling rates (after the air temperature of the freezing apparatus had cooled from ≥10 to 2°C at the rate of 0.8°C min^{-1}).

Temperature		Rate of cooling (°C min^{-1})		
air*	sample†	0.05	0.20	0.80
-6.0	-4.7	29.0 ± 2.5	33.4 ± 2.9	31.8 ± 2.2
-7.5	-6.4	35.9 ± 2.1	69.4 ± 4.5	66.4 ± 2.7
-8.6	-7.5	50.1 ± 5.7	-	-

*, programmed frost temperature, measured by the sensor of the chamber

†, frost temperature experienced by leaf discs (assessed with copper-constantan thermocouples)

4.4 Discussion

Horizontal temperature gradients of *c.* 0.5°C or less within the freezing apparatus used in these studies are comparable with those of other freezing apparatus,using either radiative or advective cooling principles (Table 4.1). Liquid cooling baths generally produce less temperature variation in sample temperatures (Raymond *et al.* 1986). However, vertical stratification of temperatures within the chamber were generally greater than horizontal variation (leaves 10 cm below the cooling coil were up to 2.0°C colder than those 5 cm above the vermiculite, a distance of *c.* 30 cm). These can be largely avoided when using whole seedlings by selecting seedlings of uniform height and positioning the temperature sensor at the level of the tissue to be assessed, or by using leaf discs, which occupy less area and are confined to one horizontal plane. Minimising temperature variation is critical, especially when small differences in frost resistance amongst genotypes or treatments are being investigated.

On average the initial freezing temperature of whole unhardened *E.nitens* seedlings (leaf material) was *c.* -3.0 to -4.5°C. This compares favourably with the range of -3 to -5°C for both hardened and unhardened *E.pauciflora* (Harwood 1980).

Spraying seedlings with water prior to frosting did not produce any noticeable increase in leaf death (Table 4.4). However, any real differences between the two treatments may have been confounded because both wet and dry leaves were frosted together, and remained in close contact. Lower levels of damage have been reported for unsprayed (non-glaucous and wettable) leaves of *E.urnigera* (Thomas and Barber 1974) and *E.pauciflora* (Harwood 1980), although the effect was much more pronounced in *E.urnigera*. Thomas and Barber (1974) suggested that dry or glaucous leaves were able to supercool to -7°C or less,and thereby escape freezing damage at certain temperatures. However, Harwood (1980) concluded that lower levels of damage to unsprayed *E.pauciflora* leaves could not be associated with their supercooling,since in his studies all leaves froze at temperatures 5°C warmer than the damaging frost temperature. He attributed the differences in damage to the slightly lower temperature recorded on sprayed leaves.

The possible problem of supercooling of leaf disc material was removed by the use of AgI and a small amount of water (see Hallam 1986). By the end of the standard frost duration (100 to 110 min) all leaf disc samples subjected to -3.8°C or less had frozen and 95% of samples subjected to frost temperatures between -3.0 and -3.8°C had frozen.

The rates of cooling (Table 4.6) and subsequent post-freeze thawing (Figure 4.5) have been shown to have large effects on the levels of damage sustained in leaf discs from partially hardened *E.nitens*. The rate of cooling also affected the level of damage in unhardened *E.nitens* seedlings (Table 4.4). Greater damage associated with rapid cooling (*c.* 1.0°C min^{-1}) compared with more gradual cooling (*c.* 3°C h^{-1}) has also been demonstrated with *E.pauciflora* (Harwood 1981) and other genera, e.g. *Thuja occientalis* (White and Weiser 1964). These different cooling rates have led to differences in estimates of frost resistance of the order of 1.5°C in *E.nitens* and several degrees in *E.pauciflora*. However, it is unlikely that the greater damage at rapid cooling rates is associated with intracellular ice formation, which is

usually lethal (Levitt 1980), since Steponkus and Wiest (1979) concluded that at cooling rates of less than 3°C min^{-1} the probability of intracellular ice formation is close to zero,for isolated cereal (rye) protoplasts. Furthermore, differences in damage with cooling rate could not be due to differences in minimum temperatures experienced by tissue,since initial and temporary exposure to freezing temperatures lower than that desired, as the result of an "overshoot" of programmed temperature, were avoided in leaf disc studies. It would appear that greater damage at rapid cooling rates may be due to a greater magnitude of stresses associated with intercellular ice formation and subsequent cellular dehydration and contraction.

The greater damage to excised *E.nitens* leaf tissue with immediate incubation of frozen tissue in distilled water (Figure 4.5), is possibly due to rapid thawing. From studies on isolated protoplasts Steponkus and Wiest (1979) proposed that freezing injury was the result of a sequence of potentially lethal stresses. One stress was a "thaw or expansion-induced dissolution of the plasma membrane which occurs when the maximum critical surface area is exceeded". Rapid thawing following a freeze may subject cellular membranes to excessive tension forces,and subsequent release of solutes into the bathing medium over and above that caused by the freeze and slower thawing.

Harwood (1980) found similar levels of damage in *E.pauciflora* seedlings frosted to -9°C, whether they were transferred to bright sunlight with an air temperature of *c.* 15°C or left to warm to 15°C over a 4 h period after the frost. The thawing rate of the *E.pauciflora* seedlings immediately transferred to 15°C was possibly similar to that of the *E.nitens* leaf discs moved from the freezing chamber and left to stand for 40 min at room temperature (*c.* 18°C).

Total electrolyte conductivity from tissue samples can be obtained by a number of methods, i.e. tissue does not have to be killed by frost *per se* (Table 4.5). Use of heat on samples for determination of total conductivity confers greater flexibility and efficiency in this part of the procedure.

Conductivity readings and estimates of relative frost resistance were affected by the size of leaf discs. The length of time for near maximum conductivities to be achieved in *E.nitens* leaf discs (*c.* 24 h) is over twice that using 6 mm diameter discs of *E.delegatensis* (Hallam 1986) or of a number of Tasmanian rainforest species (Read 1985). This is probably related to the greater mean distance across which cellular constituents have to diffuse in the larger diameter discs (Table 4.3). Whilst the trend was for larger diameter discs and/or a greater area of tissue sampled to give increased estimates of frost resistance (Table 4.3, Figure 4.3), maximum differences between disc sizes were only *c.* 0.5°C. Assessments of relative frost resistance (T50) using excised leaf tissue from a three-year-old *E.nitens* plantation (see section 6.2.4) showed that in early spring mean T50 estimates using 168 mm^2 leaf tissue from discs of 6 or 14.5 mm diameter were -6.4 and -6.9°C respectively. Hence, agreement to within 0.6°C in estimates of relative frost resistance using 6 or 14.5 mm diameter discs are maintained in *E.nitens* over a range of levels of hardiness from -4 to -7°C (Figure 4.4). This adds confidence when making comparisons with other data where similar techniques but different size discs have been used, e.g. Hallam (1986) and Raymond *et al.* (1986).

Estimates of frost resistance in eucalypts using whole seedlings and leaf discs

generally compare favourably (Hallam 1986; Raymond *et al.* 1986). The nature of the relationship between visual leaf damage (%) and the relative conductivity of bathing medium containing frosted leaf discs was determined for mature *E.nitens* leaves by frosting whole seedlings (glasshouse-grown) under standard conditions, cutting leaf discs immediately after the frost,and later assessing percentage damage to the leaves and relative electrical conductivity of the bathing medium containing discs (see Figure 4.7). The relationship indicates that a relative conductivity of 40 to 50% approximately corresponds to a leaf damage score of 50%. Similar findings have been reported for *Solanum* species (Li and Palta 1978). Harwood (1981) working with unhardened *E.pauciflora* found similar agreement in estimates of 50% leaf death when frosting whole seedlings and excised leaves,even though they were frosted in a radiation frost room and an ethylene-glycol bath respectively. Although Harwood (1981) used a resistance probe method for assessing frost damage to excised leaves (see Greenham and Daday 1957), it works on similar principles to the conductivity method,in as much as decreased resistance to low frequency alternating current and increased conductivity readings are probably both attributable to structural damage to plasma membranes with frosting (Lyons *et al.* 1979).

Frosting methods adopted for frost resistance studies using both whole seedlings and leaf discs in following chapters follow the standard procedures outlined in Section 4.2, unless otherwise stated.

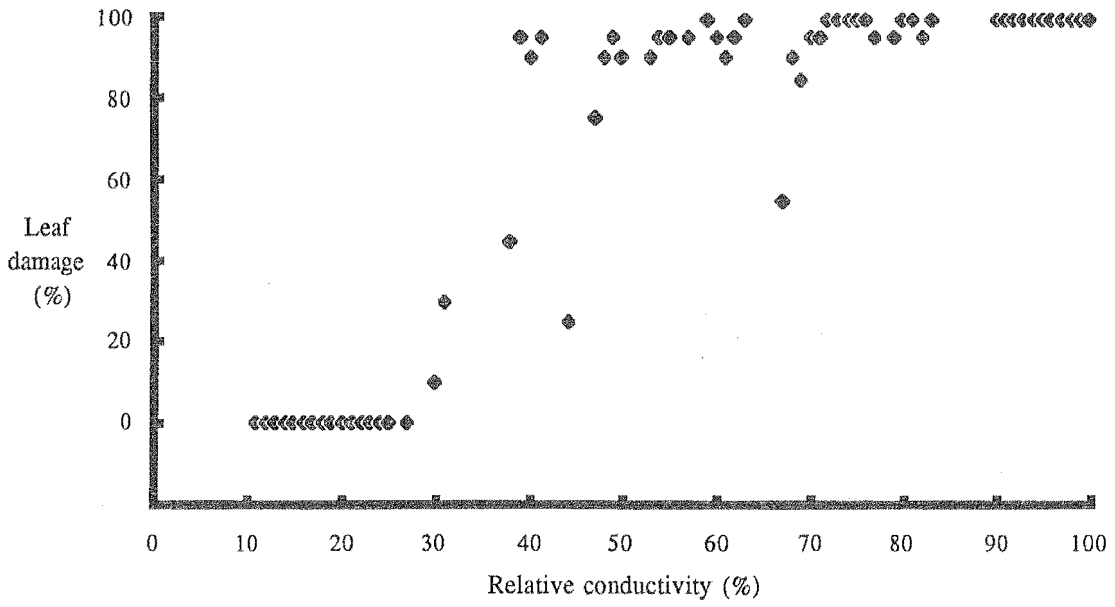


Figure 4.7 Relationships between visual leaf damage and relative electrical conductivity of the bathing medium containing leaf discs from potted *E.nitens* seedlings. Whole seedlings were frosted and discs excised after frosting.

CHAPTER 5

PHYSIOLOGICAL ASPECTS OF HARDINESS

5.1 Introduction

Frosts occur over many parts of Australia, generally with increasing severity and frequency at more southerly latitudes and higher altitudes (Turnbull and Eldridge 1983). In New South Wales, Victoria and Tasmania, the states most subject to extreme cold, screen temperatures lower than -13°C have been recorded (Hall *et al.* 1981) and absolute minima of -22°C have been recorded in New South Wales (Hall *et al.* 1981) and Tasmania (Davidson and Reid 1985). Unseasonal or particularly severe frosts cause damage to both natural populations of *Eucalyptus* (Calder 1850; Bond 1945; Paton 1983; Davidson and Reid 1985) and plantations, both within Australia (McKimm and Flinn 1979; Griffin *et al.* 1982b; Boland and Dunn 1985; Hallam 1986; Tibbits 1986) and overseas (Pilipenka 1969; Heavilin 1978; Evans 1983; Whitehead 1983; Potts and Potts 1986). However, many of the more frost resistant species are capable, as they harden during winter, of tolerating temperatures of -14°C and below (Harwood 1981; Sakai *et al.* 1981).

Over the past 20 years, studies on the frost resistance of *Eucalyptus* have consistently shown that the temperature regime imposed in the days preceeding a frost largely determines the level of damage. In particular, exposure to temperatures between 0 and 4°C for part of the day leads to increasing frost resistance for *E.regnans* (Ashton 1958; Eldridge 1969), *E.camaldulensis* (Awe and Shepherd 1975), *E.viminalis* (Paton 1972), *E.pauciflora* (Harwood 1980, 1981), *E.grandis* (Paton 1981; Eldridge *et al.* 1983), and *E.delegatensis* (Hallam 1986; Davidson and Reid 1987). However, in many of these studies only a single frost temperature has been used, which although showing a significant reduction in frost damage associated with hardening, has failed to show the full extent of hardening. Harwood (1980,1981) overcame these shortcomings by selecting the minimum temperature causing 50% leaf damage, in both whole seedlings and excised tissue, as a parameter to use in comparing levels of damage. His work indicated that *E.pauciflora* could be artificially hardened by at least 5°C.

Furthermore, studies on the rates of hardening have produced different results. Harwood (1981) found *E.pauciflora* hardened by about 1.7°C week^{-1}, whereas Paton (1980) reported the same levels of damage in *E.viminalis* following frosts of -4.0 and -8.0°C, after one and five days hardening respectively. Although different species are involved, the estimation of these two hardening rates differs by a factor of about four.

A clear understanding of the effects of growth conditions on the rates and extents of frost hardening in *Eucalyptus* species is crucial if further frost resistance studies are to be attempted. This is required to help differentiate various species or provenances (Pryor 1957a) which may be difficult to rank in an unhardened condition, e.g. *E.regnans* (Rook *et al.* 1980). In such studies, plants may need to be hardened to various levels of frost resistance to examine how the ranking of species, provenances and/or families changes with the degree of hardening attained. Furthermore, an understanding of the effects of growth conditions on the development of frost resistance is a fundamental precursor to examining the anatomical, physiological and biochemical factors which may confer increased frost resistance. These aspects remain largely unresearched in the eucalypts.

Of the eucalypts, *E.nitens* is a fast growing timber species of increasing importance in Australia (Tibbits 1986) and overseas (Darrow 1984). Studies on aspects of its frost resistance have wide practical application. This chapter examines the development of frost resistance in *E.nitens* as affected by the growth conditions imposed on the roots and shoots.

5.2 Materials and Methods

5.2.1 Frosting methods

Frost resistance was primarily determined by measuring the relative loss of electrolytes from frosted leaf discs. Closely related families of the Northern N.S.W. provenance (see Figure 3.1) were chosen for all hardiness experiments because they showed consistent hardening and were from one of the more frost resistant provenances (see Chapter 6). Methods of growing seedlings and artificial frosting were as described in Chapter 4. Usually groups of 10 seedlings were subjected to each treatment. At the commencement of the treatments, seedling height was measured and the two pairs of most recently, fully expanded leaves were identified for sampling during the course of the experiment. Seedling height was remeasured a number of times during the course of some experiments.

In addition, whole seedlings were frosted in two experiments to determine the effects of various temperature regimes on frost resistance.

5.2.2 Effects of temperature regime on hardening

Initially, assessments of frost resistance were made using whole seedlings. Three open-pollinated families were used for this experiment, viz:- N02, from the Barrington Tops (1450 m altitude) in Northern N.S.W., S15, from the relatively low altitude (1060 m) population at Brown Mountain in Southern N.S.W., and E08, from a

small population north of, and at lower altitude (935 m) than, the main Errinundra plateau population in Victoria (see Appendix A).

Sufficient seedlings of comparable height and vigour were chosen at various times and given one of the two following treatments, viz.:-

1) 12°C/3°C (growth cabinet) - seedlings placed in a growth cabinet set at 12°C day and 3°C night with 8 h artificial fluorescent and incandescent lighting of 60 μmol m^{-2} s^{-1}. Day and night temperatures varied by less than 1°C.

2) 25°C/3°C (glasshouse/cold room) - seedlings exposed to normal glasshouse conditions (light up to 1000 μmol m^{-2} s^{-1}) from 0830 h until 1630 h and then transferred to a dark cold room with a temperature maintained at *c.* 3°C. During the four weeks of treatment, day temperatures ranged from 25 to 35°C whilst night temperatures ranged from 1.5 to 4.0°C.

Treatments continued for three,seven ,14 and 28 days. Commencement of treatments was staggered so that seedlings from the four durations were under identical conditions towards the end of their respective treatment. In this way all frostings took place over a period of 11 days. Four seedlings from each family and each treatment were selected and randomly assigned to one of a number of frost temperatures, viz. -3.0, -3.5, -4.0, -4.5 or -5.0°C. Generally 24 seedlings were exposed to each frost, i.e. two treatments, three families and four seedlings. However, in a few cases some seedlings had not grown sufficiently and only three seedlings from a family-treatment combination were frosted. In all, 342 seedlings were frosted.

After the completion of the damage assessments on the above whole seedlings, frost resistance was determined on leaf discs, from new seedlings of the same open-pollinated family from the Northern N.S.W. provenance, grown at 25°C/3°C (glasshouse/cold room) and three additional treatments (Figure 5.1a). One treatment entailed keeping seedlings for 16 h nights in the unlit cold room and transferring them for 8 h to 13°C daytime conditions. The continuous 3°C treatment remained in the cold room and the artificial lighting came on automatically. A control (continuous 13°C) was also imposed. The artificial fluorescent and incandescent lighting of these three treatments was *c.* 60 μmol m^{-2} s^{-1}. Leaf, soil and ambient temperatures were monitored for at least one full 24 h period (for each of the three hardening treatments and the control) using copper-constantan thermocouples connected to a portable recorder (Figure 5.2).

After 14,28 and 56 days of treatment,relative frost resistance was evaluated using leaf discs frosted to a range of temperatures. Sampling always took place during daytime conditions.

The seedlings receiving the continuous 3°C treatment displayed visual symptoms of water stress by four weeks. Leaf relative water status (RWC) of the seedlings under this treatment was determined after four and eight weeks as follows. Three discs (6 mm diameter) were taken from the leaves where discs had been sampled for frosting, and quickly weighed (to 0.1 mg) for fresh weight (F.Wt) before floating on distilled water in

sealed vials. After 24 h the surface of the tissue was carefully dried with paper towels, the tissue weighed for turgid weight (T.Wt) and subsequently reweighed after oven drying for 48 h at 90°C for oven dry weight (O.D.Wt). RWC was calculated (after Weatherley 1950) as,

RWC% = [(F.Wt - O.D.Wt) ÷ (T.Wt - O.D.Wt)] x 100

5.2.3 Effects of photoperiod and daily duration of 2°C on hardening

Twenty glasshouse-grown seedlings from each of two families were sorted into four groups of 10 seedlings and each group allocated to one of four treatments,in a two photoperiod by two daily durations of 2°C factorial design (Figure 5.1b). The artificially lit photoperiods (*c.* 300 μmol m^{-2} s^{-1}) and the time at 2°C were either 8 or 16 h. The alternate temperature was 20°C. After 25 and 46 days relative frost resistance was evaluated using leaf discs. Seedlings were kept under uniform conditions during disc sampling by extending the common 2°C dark period.

5.2.4 Effects of photoperiod and temperature regime on dehardening

Dehardening was initially examined in seedlings previously grown for eight weeks under the various day temperatures with 3°C nights (Figure 5.1a). Seedlings from the continuous 3°C and 13°C/3°C treatments were placed with the control (continuous 13°C), whilst seedlings from the 25°C/3°C (glasshouse/cold room) treatment were left in the glasshouse (mean minima 15°C). Relative frost resistance was determined during daytime conditions after two and four weeks of dehardening.

A further experiment examined in detail the effects of photoperiod and temperature regime on dehardening. Fifty seedlings (representing five families) were hardened using the 25°C/3°C treatment for eight weeks, at the end of which their frost resistance was evaluated using leaf discs. Seedlings were then transferred to one of five dehardening treatments, each with 18°C days and artificial lighting (*c.* 100 μmol m^2 s^{-1}) but variable night temperatures and/or photoperiods (see Figure 5.1c). After seven days, seedlings with 18°C shoots and 3°C roots (treatment E) were transferred to a treatment with roots and shoots at 18°C and an 18 h photoperiod (treatment F). Relative frost resistance was determined at 7,14, 25 and 40 days after the imposition of dehardening treatments.

To examine the effects of day temperature on dehardening,20 seedlings which had been hardened to -5.9°C were subjected to one of two daytime temperatures, viz., 20°C and 14°C, maintained for 10 h. Night temperatures were 6°C. Relative frost resistance was determined 12 days after the imposition of dehardening treatments.

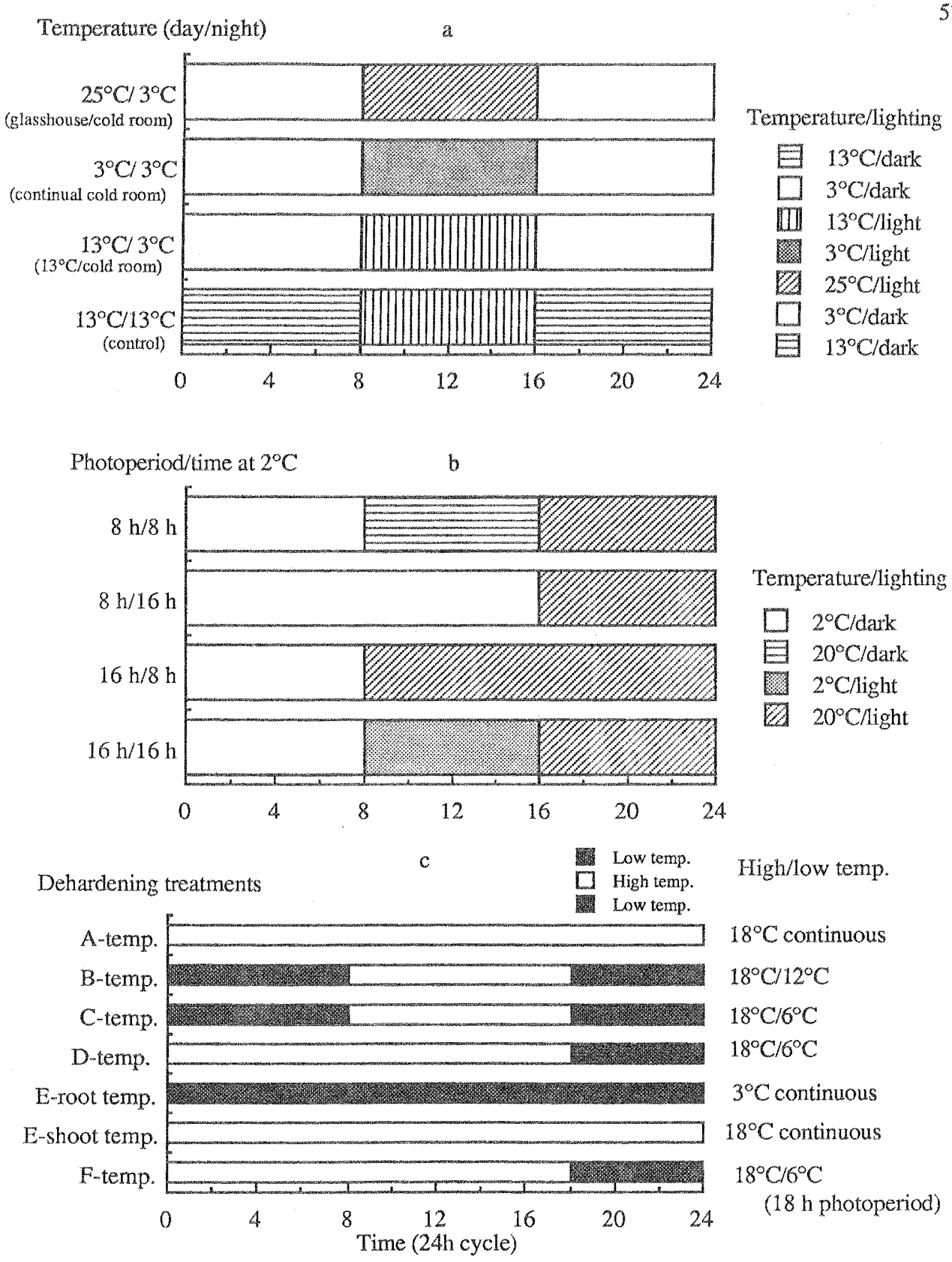


Figure 5.1 Photoperiod and temperature regimes used when examining (a) effects of temperature regime on hardening (8 h photoperiod), (b) effects of photoperiod and daily duration of 2°C on hardening, and (c) effects of photoperiod and temperature regime on dehardening, (10 h photoperiod from 0800 to 1800 h unless otherwise stated).

5.2.5 Root- shoot interactions and hardiness

The three following experiments were designed to investigate the influence of root temperature on the degree of hardening attained in *E.nitens*.

Firstly, seedlings were grown under the continuous 3°C treatment as described above but with one of two root treatments, viz.,

1) cold roots- roots untreated as in the previous experiment (mean temperature *c.* 3°C, Figure 5.2b)

2) heated roots- seedlings placed on a metal light box which transferred heat to the pots and roots, keeping soil temperatures at *c.* 8°C (ranging from 6 to 10°C from the top to the base of the pots respectively, Figure 5.2b).

Secondly, this experiment was repeated with root heating increased to *c.* 14°C (top of the pots 10°C and base of the pots 18°C). A treatment with 18°C shoots with 3°C roots was also implemented (treatment E as in Figure 5.1c) by placing shoots in an oven which continuously maintained leaf temperature at *c.* 18°C whilst roots were outside the oven and were effectively maintained at 3°C. The warm shoots with cold roots treatment was discontinued after 28 days due to poor health. Thirdly, seedlings were grown for eight weeks at 25°C/3°C (glasshouse/cold room) with one of the two root treatments (3°C or heated to *c.* 14°C) imposed during the 16 h night.

Leaf discs were taken during daytime conditions at various durations of treatment to determine frost resistance. Leaf relative water status was also calculated at various times in the second experiment.

5.3 Results

5.3.1 Effects of temperature regime on hardening

The frost temperatures which correspond to mean leaf damage scores of 50% for whole seedlings were evaluated and are presented in Table 5.1. Determination of the temperature giving a mean leaf damage score of 50% was difficult in the 25°C/3°C (glasshouse/cold room) treatment on two occasions because of an irregular trend in the relationship between percentage leaf damage and frost temperature, i.e. mean frost damage score fell below 50% with increasing severity of frost. In both cases the temperature causing a mean frost damage score of 50% was estimated as the mean of the two temperatures at which leaf damage increased to 50%.

Conditioning treatment was the only significant factor ($P<0.05$) with the 25°C/3°C treatment having lower (more negative) frost temperatures corresponding to mean leaf damage scores of 50% than the 12°C/3°C treatment, viz., seedlings grown at 25°C/3°C were more frost resistant than those grown in the 12°C/3°C treatment. The areas

under the plots of mean leaf damage against frost temperature, from -3.0 to -4.0°C and -3 to -4.5°C, were integrated and again conditioning treatment was found to be the only significant factor ($P<0.05$). However, there were trends for increasing frost resistance with increasing duration of hardening for the more resistant Northern N.S.W. family in the 25°C/3°C (glasshouse/cold room) treatment.

The main differences in growth conditions between the treatments were, firstly the higher daytime temperature and light intensities of the 25°C/3°C (glasshouse/cold room) treatment, and secondly, the more rapid decrease to the low night temperature of the 25°C/3°C (glasshouse/cold room) treatment, and hence longer period of time at low temperature. The change in leaf temperature from day to night was almost instantaneous in seedlings taken from the glasshouse to the cold room, whereas in the cabinet (12°C/3°C) it presumably took much longer as the air temperature took about 7 h to cool from 12 to 3°C. One or more of these factors contributed to the greater frost resistance of the seedlings grown at 25°C/3°C. To avoid these problems of long delays in cabinets reaching 3°C night temperatures, all further temperatures of *c.* 3°C were imposed by either transferring seedlings from their daytime location to a cabinet already at *c.* 3°C or by using cabinets which were capable of lowering their temperatures from 20 to 2°C in less than 30 min (treatments in Figure 5.1b).

Table 5.1 Frost temperatures (°C) resulting in 50% leaf damage to whole *E.nitens* seedlings being hardened at two temperature regimes. Mean maximum temperature and photoperiod were coincident and lasted 8 h.

Temperature regime	Duration of treatment (days)			
	3	7	14	28
Northern N.S.W family				
12°C/3°C	-3.2	-3.4	-3.4	-3.2
25°C/3°C	-3.2	-3.6	-4.4	-5.2
Southern N.S.W family				
12°C/3°C	-3.1	-3.0	-3.0	-3.4
25°C/3°C	-3.4	-3.4	-3.9	-3.8
Errinundra family				
12°C/3°C	-3.3	-3.2	-3.0	-3.3
25°C/3°C	-3.4	-3.2	-3.5	-3.4

The changes in temperature resulting in a relative conductivity reading of 50% (T50) with time after imposition of the four temperature regimes (see Figure 5.1a) are shown in Figure 5.3. Throughout the 56 days of hardening the control showed no real change in T50 whilst the T50 values for the other three treatments decreased with time, indicating increased frost resistance. After only 14 days, all three treatments were significantly different from the control ($P<0.001$), though there were no significant differences amongst these treatments ($P>0.05$). The relative ranking of the treatments in order of decreasing T50 was the same at 14, 28 and 56 days, i.e. continuous 13°C>continuous 3°C>13°C/3°C>25°C/3°C. The continuous 3°C treatment was significantly ($P<0.01$) less hardy than the 13°C/3°C and 25°C/3°C treatments at 28 and 56 days. The differences between the 13°C/3°C and the 25°C/3°C treatments were significant ($P<0.05$) at 28 days only.

One family of *E.nitens*, when grown under 25°C/3°C conditions, received 50% leaf death in whole seedlings (Table 5.1) and 50% loss of cellular electrolytes from leaf discs (Figure 5.3), at temperatures of -4.4°C and -4.3°C respectively after 14 days, and -5.2 and -5.8°C respectively after 28 days hardening, suggesting that the estimates of frost resistance by the two techniques are comparable. This is supported by the findings of Hallam (1986) using the same equipment with *E.delegatensis*.

Leaf temperatures generally responded much more rapidly to the transitions from the 3°C cold to daytime conditions, and *vice versa* (normally less than 5 min), than soil temperatures (Figure 5.2). In fact, the soil temperature of seedlings in the 25°C/3°C and 13°C/3°C treatments only equilibriated with ambient temperature about three and seven hours respectively after being removed from 3°C. Root and leaf temperature of seedlings kept in the continuous 3°C treatment remained in equilibrium with the air temperature.

The only seedling mortality occurred in the continuous 3°C treatment, in which three of the 10 seedlings wilted beyond recovery, despite being well watered. During the eight weeks of hardening, overall mean seedling height growth was quite small at 2.9 cm. There were significant differences amongst treatments ($P<0.01$) with the 25°C/3°C treatment growing the most (6.1 cm) and the continuous 3°C treatment growing the least (0.8 cm).

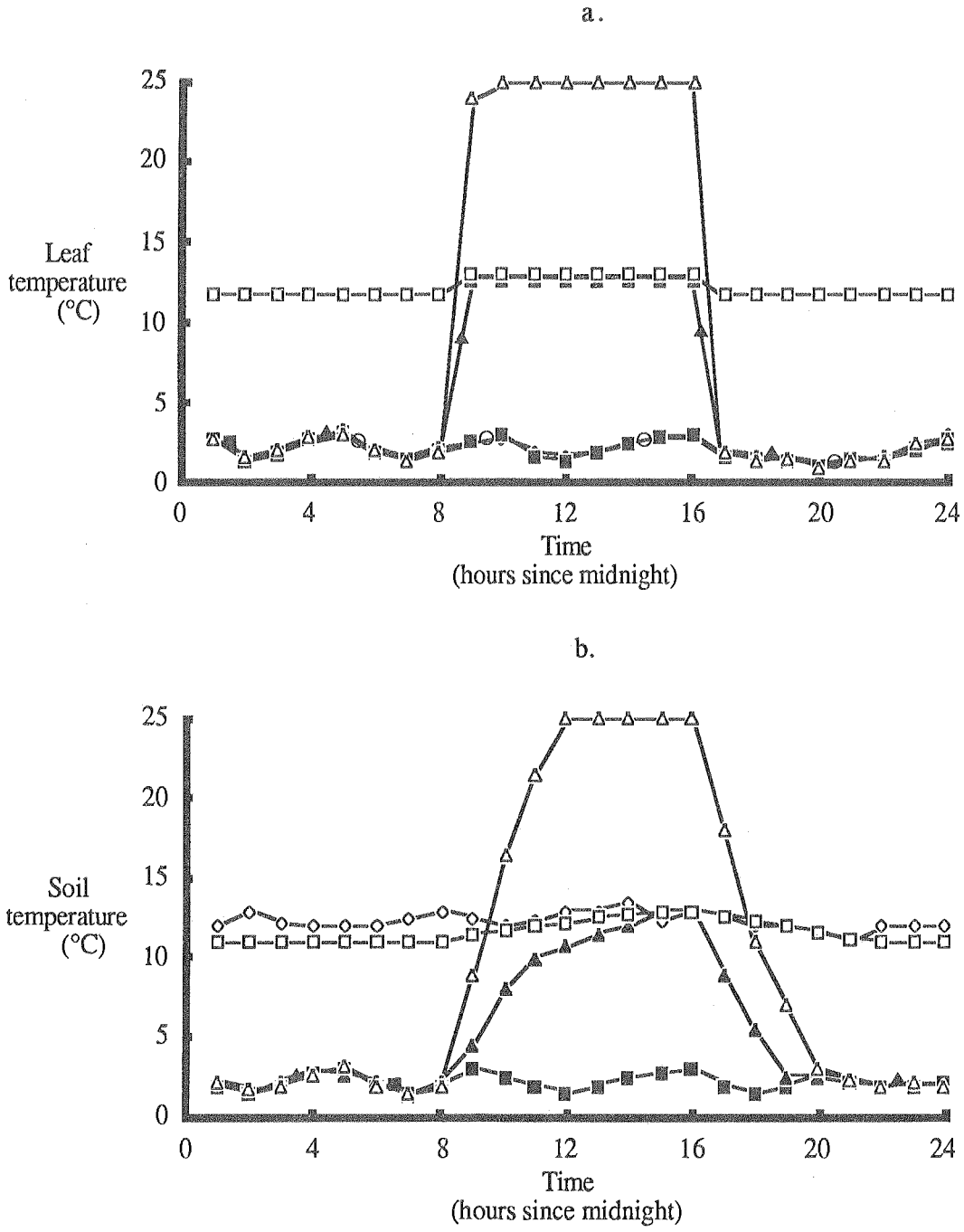


Figure 5.2 Mean diurnal variation in leaf and soil temperature of hardening *E.nitens* seedlings. Growth conditions are 8 h photoperiod with day/night temperatures of, (□) 13°C constant, (■) 3°C constant with cold roots, (○) 3°C constant with heated roots, (▲) 13°C day/3°C night, and (△) glasshouse (*c.* 25°C)day/3°C night.

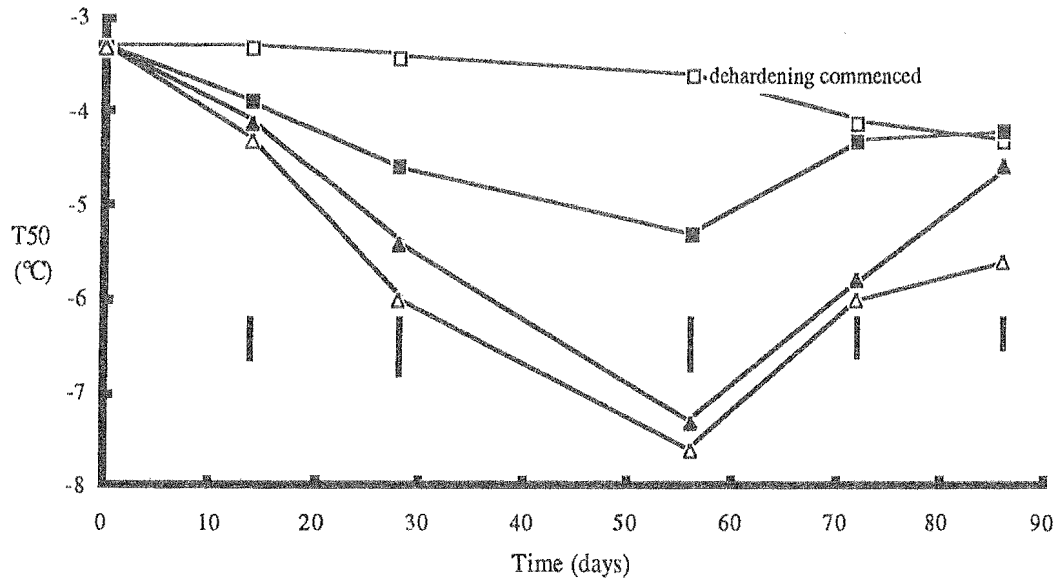


Figure 5.3 Changes in frost temperature causing 50% leakage of cellular electrolytes (T50) from leaf discs of hardening and dehardening *E.nitens* seedlings. Hardening treatments were 8 h photoperiod with day/night temperatures of, (□) continuous 13°C, (■) continuous 3°C, (▲) 13°C/3°C, and (△) 25°C/3°C. Dehardening commenced at 56 days using treatments with day(8 h)/night temperatures of, (□ ■ ▲) continuous 13°C and (△) 25°C/15°C. Vertical bars are L.S.D. (P<0.01).

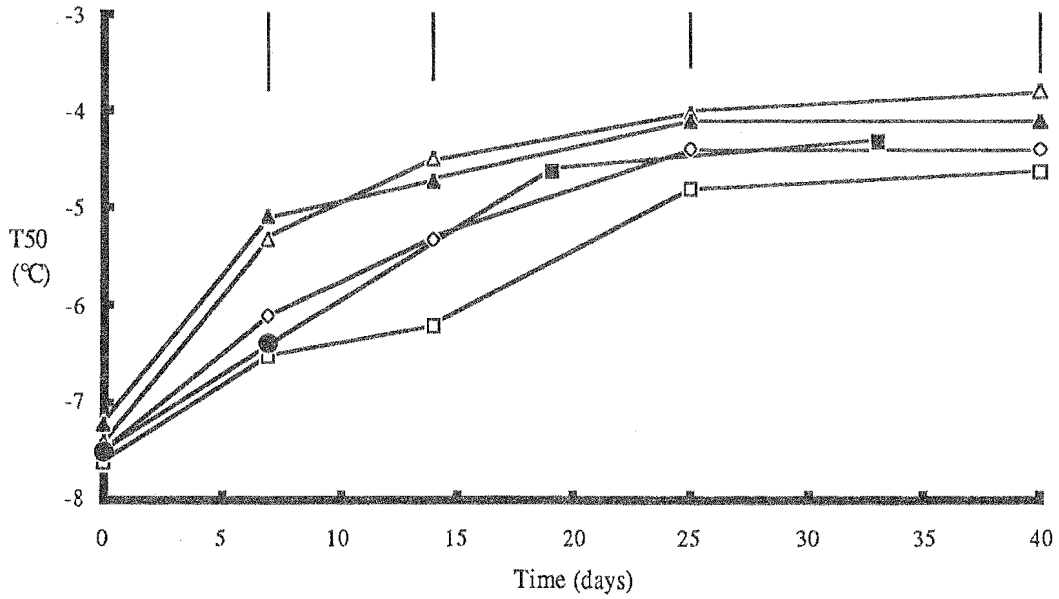


Figure 5.4 Changes in T50 of dehardening *E.nitens* seedlings. Day/night temperatures of treatments (10 h photoperiod and 14 h at night temperature unless otherwise stated) as given in Figure 5.1c are:- 18°C/18°C (△), 18°C/12°C (○), 18°C/6°C (□), 18°C/6°C(only 6 h at 6°C) (▲), shoots continuous 18°C/roots continuous 3°C (●), and 18°C/6°C(18 h photoperiod, only 6 h at 6°C) (■). Vertical bars are L.S.D. (P<0.01).

5.3.2 Effects of photoperiod and daily duration of 2°C on hardening

Table 5.2 summarizes the T50 values at 25 and 46 days for seedlings grown under the two photoperiods and two daily durations of 2°C. At both times of assessment there were significant differences between the two daily durations of 2°C ($P<0.001$) but not the photoperiods ($P>0.05$). After 46 days, 16 h day^{-1} at 2.0°C, produced seedlings that were effectively 1.2°C hardier than seedlings grown for only 8 h day^{-1} at 2.0°C. These results clearly show that the level of frost resistance in *E.nitens* foliage is largely controlled by the length of time that shoots are exposed to low temperatures. The two families of *E.nitens* used, differed by 0.3°C on average in their level of frost resistance which was significant ($P<0.01$).

Table 5.2 Frost temperatures (°C) resulting in 50% leakage of cellular electrolytes from leaf discs of *E.nitens* seedlings. Hardening was at 2.0°C with the alternate temperature being 20°C. L.S.D. ($P<0.01$) between daily durations of 2°C are 0.4°C at 25 days and 1.0°C at 46 days.

Photoperiod (h)	Daily duration of 2°C (h) 8	16	Mean
25 days hardening			
8	-4.4	-4.7	-4.6
16	-4.4	- 5.1	-4.8
Mean	-4.4	-4.9	-4.7
46 days hardening			
8	-5.2	-6.6	-5.9
16	-5.5	-6.5	-6.0
Mean	-5.4	-6.6	-6.0

5.3.3 Effects of photoperiod and temperature regime on dehardening

Figure 5.3 shows the loss of frost resistance with time following the cessation of the three hardening treatments (Figure 5.1a) after eight weeks. The relative increase in T50, i.e. dehardening, was 1.0 to 2.0°C week^{-1}. After 21 days of dehardening, seedlings formerly grown under the continuous 3°C and 13°C/3°C treatments possessed a similar level of hardiness to those grown in the continuous 13°C treatment. These three groups of seedlings were in the same cabinet and all developed callus on their leaves (visual observations). Consequently, after 21 days of dehardening only the 25°C/3°C treatment was still significantly different ($P<0.01$) from other treatments.

The effects of dehardening photoperiods and temperature regimes on relative frost resistance are shown in Figure 5.4. Dehardening with time appeared asymptotic to T50 values of -3.8 to -4.6°C. Overall, dehardening was roughly twice as rapid as hardening, e.g. after 25 days of dehardening the mean T50 had fallen from -7.5°C to -4.4°C (*c.* 0.9°C week^{-1}) compared with the rise in T50 to -7.5°C from -3.2°C (Figure 5.3) after eight weeks of hardening (*c.* 0.5°C week^{-1}). After only seven days of dehardening there were large and highly significant ($P<0.001$) differences amongst treatments. From this time onwards, treatment (night temperature for 14 h unless otherwise stated) ranking in order of increasing T50 (increasing rate of dehardening) was always 6°C≤12°C<6°C(6h)=18°C (see Figure 5.1c for treatment details). This means that the lower the night temperature the slower the initial dehardening that takes place, with the 6°C night temperature (14 h) exhibiting the slowest initial dehardening. The rapid dehardening with only 6 h at 6°C (treatment D, Figure 5.1c) indicates that the rate of dehardening is affected by the whole temperature regime, i.e. a period of low temperature *per se* does not necessarily reduce dehardening.

Keeping the roots cold (Treatment E, Figure 5.1c) for the first seven days following hardening did not effectively stop dehardening, i.e. the mean level of hardiness changed from -7.5 to -6.4°C (Figure 5.4). However, the cold (3°C) roots did result in a significantly ($P<0.01$) slower rate of dehardening than that of seedlings with roots and shoots subjected to 18°C, viz., T50 changing from -7.5 to -5.2°C. By interpolation, the rate of dehardening from a mean T50 of -6.4°C appeared to be the same in the two treatments with 18 h at 18°C and 6 h at 6°C, at either 10 or 18 h photoperiods (treatments D and F, Figure 5.1c). This indicates that as for hardening (Table 5.2) there were no photoperiodic effects on dehardening at the range of frost resistances, temperatures and lightings used.

The extent of dehardening was also significantly ($P<0.01$) affected by day temperature. Seedlings grown with common 6°C (14 h) nights, dehardened by only 0.4°C if grown under 14°C days, whereas those grown under 20°C days dehardened by

1.4 °C. This 1.4°C reduction in frost resistance (T50) compares favourably with the 1.1°C dehardening of the 18°C day/ 6°C night (Figure 5.4) from a similar starting temperature of -5.9°C. Consequently, increasing day and/or night temperatures in the range of 6°C to 20°C results in increasing dehardening.

5.3.4 Root-shoot interactions and hardiness

The changes in relative frost resistance with time are shown in Table 5.3 for the three separate experiments which examined hardening responses with roots and/or shoots under differential temperature regimes. Three main points can be drawn from the results. Firstly, under continuous 3°C air temperatures there were no significant differences ($P>0.05$) on any occasions between relative frost resistance of the heated and unheated root treatments, irrespective of whether roots were heated to 8 or 14°C (Table 5.3). Secondly, maximum hardiness appears to be achieved somewhere between 28 and 56 days of the continuous 3°C (shoot) conditioning. Thirdly, there were no significant difference over 56 days ($P>0.05$) in the mean relative frost resistance of seedlings grown with roots at 3°C or heated to 14°C for the 16 h night phase of the 25°C/3°C treatment (glasshouse/cold room).

Seedlings grown for 28 days with 18°C shoots and 3°C roots did not differ significantly in mean level of hardiness from seedlings with 3°C shoots and 3°C, or 8 to 14°C roots (Table 5.3). However, the seedlings with 18°C shoots and 3°C roots experienced leaf wilt in the first few days of treatment (see also Paton *et al.* 1979) and had suffered excessive leaf fall and some mortality by the end of 28 days of treatment.

The relative water content of leaf material and its correlation with relative frost resistance or ionic leakage from unfrosted discs, are shown in Table 5.4 and Figure 5.5, for seedlings grown under continuous 3°C ambient conditions. Seedlings with 14°C roots were characterised by significantly ($P<0.01$) higher relative water status than seedlings with 3°C roots. This was also reflected in the 30 or 40% mortality which occurred in seedlings grown for 56 days or more with roots continually at 3°C (in three separate experiments), whilst no seedlings died if their roots were continually heated. Seedling death occurred in each instance where a leaf relative water status below 50% was recorded.

Table 5.3 Frost temperatures (°C) resulting in 50% leakage of cellular electrolytes (T50) from leaf discs of hardening *E.nitens* seedlings. Seedlings were kept in a cold room (3°C ambient temperature) for 16 or 24 h with various root/shoot temperatures. No values within an experiment at a given duration are significantly different (P>0.05).

Day conditions (8 h)*	Duration (days)	Root/shoot temperatures**			
		3°C/3°C	8°C/3°C	14°C/3°C	3°C/18°C†
3°C	28	-5.0	-5.3	-4.7	-4.6
	56	-5.8	-5.8	-5.8	-
	77	-5.6	-5.6	-5.6	-
25°C	56	-7.5	-	-7.5	-

*, ambient temperatures of 3°C in a cold room or 25°C in a glasshouse

**, during the period in the cold room

†, shoots enclosed in an oven

Table 5.4 Leaf relative water contents (%) of *E.nitens* seedlings hardening under continuous 3°C ambient conditions. Values with similar letters are not significantly different (P<0.01).

Root/shoot temperature	Duration of treatment		
	28 days	56 days	77 days
3°C/3°C	80b	70b	75b
14°C/3°C	-	90a	91a

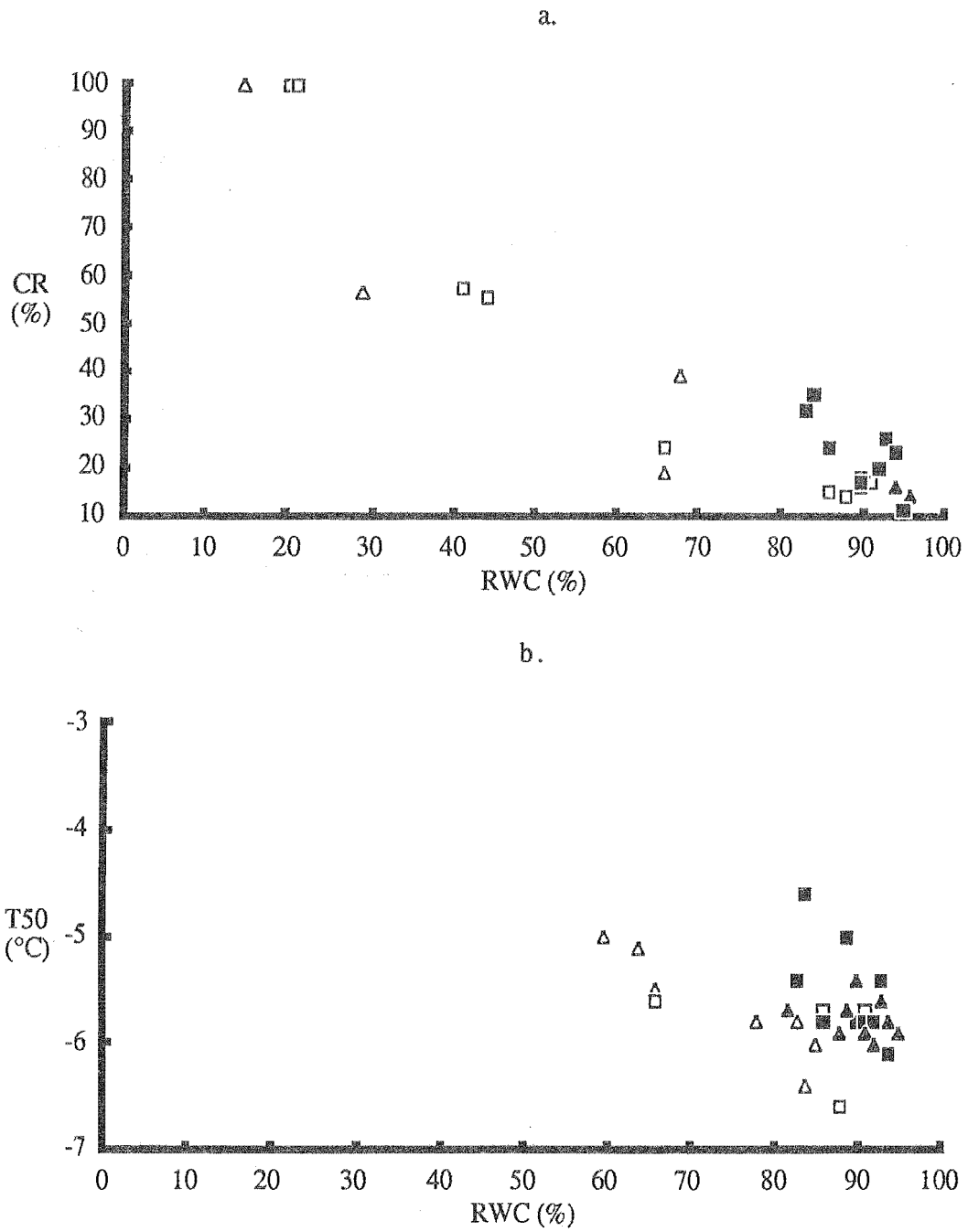


Figure 5.5 Relationships between leaf relative water content (RWC) and (a) relative electrical conductivity of bathing medium containing unfrosted discs (CR) , and (b) frost temperature causing 50% leakage of cellular electrolytes (T50) from leaf discs, of potted *E.nitens* seedlings under the continuous 3°C ambient conditions. Symbols are :- seedlings with cold (3°C) roots after (△) 56 days and (□) 77 days, and seedlings with roots heated to 14°C after (▲) 56 days and (■) 77 days.

The relationship between relative conductivity of unfrosted discs and water status (Figure 5.5a) shows that as water deficit increases the ionic leakage from discs also increases and,at leaf relative water contents of 50% and less,the relative conductivity from unfrosted discs was already above 50%, i.e. above the intercept usually chosen to determine relative frost resistance (T50) from the relationship between relative conductivity and frost temperature. This indicates that cellular membranes systems had possibly been damaged as a result of dehydration stresses (see Gaff 1980). Electron microscopy studies with *Solanum* species have shown damage to cellular membranes following frosting (Li and Palta 1978). Therefore, it is not possible using the leaf disc method to determine frost resistance of seedlings once their leaf water status becomes critical. The relationships between frost resistance and water status (Figure 5.5b) were subjected to linear regression analysis to test the significance of slopes. The slope of the regression for seedlings with cold roots was significantly different from zero ($P<0.01$), and indicated that the greater the leaf water deficit the lower the frost resistance. However, for seedlings with heated roots the slope of the linear regression was not significant, indicating no correlation between frost resistance and water status. This appears to contradict the findings that seedlings with cold roots were characterised by lower leaf water contents,yet similar frost resistance to seedlings with warm roots. It is likely that the apparent correlation between frost resistance and water status for seedlings with cold roots is the consequence of the way that the assessment of frost resistance is based on relative conductivity, and relative conductivity of unfrosted discs is affected by water status, i.e. frosted tissue with increasing water deficit may leak 50% of cellular electrolytes at milder temperatures because they have higher relative leakage of electrolytes at undamaging temperatures.

5.4 Discussion

The growth treatment, and particularly the temperature regime prior to frosting ,is crucial to the level of hardiness obtained in *E.nitens*. In this study, a number of separate experiments have shown that exposure to temperatures of between 1.5 and 4.0°C for at least some part of the day/night cycle allows hardening to develop in *E.nitens*. This confirms the findings with many other *Eucalyptus* species, e.g. *E.regnans* (Ashton 1958; Eldridge 1969), *E.camaldulensis* (Awe and Shepherd 1975), *E.viminalis* (Paton 1972), *E.pauciflora* (Harwood 1980,1981), *E.grandis* (Eldridge *et al.* 1983; Paton 1981), and *E.delegatensis* (Hallam 1986). Moreover, increasing both the daily amount and total duration (days) of hardening temperature generally results in a greater level of hardiness in *E.nitens*. A 16 h daily period of 2°C over 42 days hardened *E.nitens* seedlings by 50% more than a 8 h daily period, i.e. -6.5 c.f. -5.4°C from *c.* -3.2°C unhardened (Table 5.2).

This may explain the superior frost resistance of whole seedlings grown under the 25°C/3°C treatment compared with a 12°C/3°C treatment (Table 5.1), viz., the seedlings transferred from the glasshouse to the cold room at night had a greater effective time at *c.* 3°C compared with those kept in a cabinet, i.e. 16 and 9 h respectively.

However, increasing the daily exposure to low temperature hardening (3°C cold room) to the maximum of 24 h was found to be detrimental to seedling health and to restrict further hardening after more than four weeks exposure. Whilst heating the seedling roots to either 8 or 14°C significantly improved seedling health under continual cold room conditions (Table 5.4), it did not confer any increased ability to harden under either 3°C artificially lit or glasshouse day conditions (Table 5.3). The poor seedling health in the continual 3°C conditions was, therefore, attributable to cold and metabolically inactive roots. Paton (1980) did not report deteriorating seedling health in *E.viminalis* grown at constant 2.0°C, but his work reports on frost resistance for only up to 15 days of hardening. It therefore appears that an 8 h period of warm (13°C+) day temperatures confers some level of metabolic activity that is required for substantial hardening to take place. However, the interaction between seedling health and the hardening response appears to be complex as evidenced by the slight hardening of seedlings grown with 18°C shoots and 3°C roots (Table 5.3) and by the increased hardiness of seedlings which developed callus in the continuous 13°C treatment (Figure 5.3). Callus tissue is capable of hardening in a number of genera (see Sugawara and Sakai 1978).

There have been few comparisons between the use of whole seedlings and excised leaf material to assess the frost resistance of *Eucalyptus* species. The present study shows that hardening the same family of *E.nitens* under an identical treatment results in temperatures producing 50% leaf death in whole seedlings (Table 5.1) and 50% loss of cellular electrolytes from leaf discs (Figure 5.3) of -4.4 and -4.3°C respectively after 14 days and -5.2 and -5.8°C respectively after 28 days hardening. In addition a relative conductivity of 40 to 50% approximately corresponds to a leaf damage score of 50% for whole seedlings (Chapter 4, Figure 4.7). Similar findings have been reported for *Solanum* species (Li and Palta 1978). Harwood (1981) working with unhardened *E.pauciflora* found similar agreement in estimates of 50% leaf death when frosting whole seedlings and excised leaves, even though they were frosted in a radiation frost room and an ethylene-glycol cooling bath respectively. Although Harwood (1981) used a resistance probe method for assessing frost damage to excised leaves (see Greenham and Daday 1957), it works on similar principles to the conductivity method in as much as decreased resistance to low frequency alternating current and increased conductivity readings are both attributable to structural damage to cellular membranes with frosting (Lyons *et al.* 1979).

This ability to detect hardening,whether using whole seedlings or excised leaf material,reinforces the many advantages of using excised leaf material, viz., it is more

efficient in both time and space; it is more flexible, since material from plants of virtually any size and from various locations can be used; it allows results to be obtained more rapidly; it does not destroy plants which may be extremely valuable, e.g. artifically produced hybrids.

Three separate experiments using leaf discs showed *E.nitens* seedlings hardening by about 0.5°C week^{-1}. Leaf disc studies on the seasonal variation in frost resistance of three year-old *E.nitens* plantations have shown that the rate of hardening over winter, deduced from frost resistance levels in late autumn and mid-winter, ranged from 0.4 to 0.6°C week^{-1} for eight families representing the full range of provenances (see Chapter 6, Table 6.6). This compares with a similar rate in *E.delegatensis* (Hallam 1986) and slightly higher in *E.gunnii* and *E.perriniana* (see Table 7.5) and a higher rate of *c.* 1.7°C week^{-1} in *E.pauciflora* (Harwood 1981). These hardening rates are in stark contrast to those reported for whole *E.viminalis* seedlings by Paton (1980), who found that five days of 2.0°C hardening reduced damage at -8.0°C to the same level as -4.0°C after one days hardening (a rate of at least 4°C week^{-1}). Neither the work of Harwood (1980,1981) nor the present study show such rapid hardening using either whole seedlings or leaf tissue.

There is a great range in the rates of hardening at comparable low temperature treatments in plant species from a number of genera (Table 1.1). *S.commersonii* and *S.acaule* hardened by about 0.7°C week^{-1} (Chen and Li 1976) whilst five Tasmanian rainforest tree species {Read (1985) using the same technique as Hallam (1986)} were found to harden at rates between those reported here for *E.nitens* and those of Harwood (1981) for *E.pauciflora*. However, hardening rates exceeding 3°C week^{-1} have been found in herbaceous crops, e.g. *Spinacea oleracea* L. (Klosson and Krause 1981) and winter wheat (Gusta *et al.* 1982), and Northern Hemisphere forest trees, e.g. *Pinus sylvestris* L. (Smit-Spinks *et al.* 1985).

Like the hardening process, the dehardening process is markedly influenced by the temperature regime in *E.nitens*. Previous research has shown dehardening occurs with constant temperatures at least 5°C above those used to promote hardening, e.g. in *E.pauciflora* (Harwood 1981). However, the present study shows as little as 3°C above hardening temperatures is effective. This study also demonstrates that initial rates of dehardening increase with either increased day and/or night temperature irrespective of photoperiod. The maximum rates of dehardening detected in *E.nitens* were *c.* 2.0°C week^{-1} (Figure 5.4), and are four times that of hardening in *E.nitens* (Figure 5.3) and twice that of dehardening reported in *E.pauciflora* (Harwood 1981). Should commencement of growth after winter and the dehardening process be linked, the greater rate of dehardening in *E.nitens* may confer earlier growth which may help explain why its growth rate is more rapid and continuous than many other *Eucalyptus* species in many parts of Tasmania (C.L.Beadle pers. comm.).

Paton *et al.* (1979) found that inactive roots delayed the dehardening process in

well hardened *E.urnigera*. Their studies, however, are based on a single frost temperature which, although indicating differences between treatments, does not show the extent of the differences. Examinations of the effects of cold roots on dehardening in *E.nitens* has shown that although seedlings with roots colder than shoots (3 c.f. 18°C) remain significantly more frost resistant than seedlings dehardening with roots and shoots at constant warm temperatures (18°C), keeping the roots cold does not stop dehardening *per se*. These seedlings deharden by the same extent as those with a 14 h period of 6°C night after an 18°C day.

Both the hardening and dehardening processes appear to display quantitative rather than threshold responses to temperature regime. Night temperatures of 10°C have conferred some hardening in *E.viminalis* (Paton 1972). In *E.nitens*, day/night temperatures of 12°C/8°C produced significantly ($P<0.05$) more frost resistant seedlings than 16°C/12°C, though the extent of hardening was been much less than that with 4°C night temperatures (Table 5.5). However, night temperatures of 6°C, i.e. only *c.* 3°C warmer than those which promote considerable hardening, are unable to maintain high levels of hardiness in *E.nitens* (Figure 5.4). Therefore, it would appear that lowering the temperature regime leads to increasing, albeit non-linear, levels of hardiness, whilst any subsequent increase in the temperature regime may lead to subsequent dehardening.

No photoperiodic responses of hardening nor dehardening were found in the present study. Eldridge (1968, 1969) repeatedly found, at a range of damaging temperatures, that high altitude populations of *E.regnans* hardened significantly more when grown under shorter (8 h) rather than long (16 h) photoperiods. He also reported that seedlings from a single family of *E.nitens* from Mt.Erica in Victoria when frosted to -5.0°C, after four weeks hardening at 9°C day (8 h)/4°C night, were less damaged if grown at an 8 h rather than a 16 h photoperiod (Eldridge 1969). Although the differences were significant ($P<0.05$) the mean levels of damage were quite low at 3 and15% respectively. At these levels of damage, death to a single seedling from one treatment could dramatically influence the overall significance of means. Photoperiodic responses were also not evident in *E.delegatensis* seedlings (Eldridge 1969). Paton (1981) found that whole *E.viminalis* seedlings grown for 10 h day^{-1} at 12°C/14 h day^{-1} at 0.5°C hardened sufficiently after only two days so that damage at -6.6°C was reduced from maximum to virtually nil, irrespective of whether grown at either 10 h or 20 h photoperiods. However, any differences in relative frost resistance between photoperiodic treatments with further exposure to hardening treatments could not be determined,because the single frost temperature used only produced minimal damage after two days. In contrast, the present study clearly showed for *E.nitens* no significant differences between the relative frost resistance of the photoperiodic treatments, at a range of damaging temperatures and at a number of levels of hardiness. The *E.nitens* seedlings studied here were from the highest altitude populations found. The small effects of photoperiod on hardening in *Eucalyptus* are in marked contrast to the large effects of

short days on hardening in many northern hemisphere species (see Smit-Spinks *et al.* 1985).

This work demonstrates that the frost resistance of *E.nitens* can be increased by at least 4°C under controlled environment conditions and that the temperature regime to which the shoot is exposed is the major factor controlling the level of hardiness. Such findings enable genetic variation in frost resistance to be confidently examined at a range of levels of hardiness by artificially hardening seedlings. In addition, these findings lay the foundation for studies of the anatomical, physiological and biochemical factors which may confer increased frost resistance.

Table 5.5 Frost temperatures (°C) resulting in 50% mean leaf damage and 50% death of stem height to whole *E.nitens* seedlings after 16 days growth under three treatments. Treatments consisted of 12 h day^{-1} at either 16,12 or 8°C (of which the first 11 h coincided with a photoperiod at 100 µmol m^{-2} s^{-1}) followed by a 12 h dark period at 12,8 or 4°C respectively. Each frosting contained 24 seedlings (comprising six seedlings from each provenance) from one of the three treatments and followed standard procedures. L.S.D. ($P<0.05$) between treatments are 0.2°C for both leaf and stem damage.

Provenance	Treatment 16°C/12°C[A]	12°C/8°C[B]	8°C/4°C [C]
Leaf damage			
Rubicon	-3.7	-3.8	-4.5
Toorongo	-3.4	-3.5	-4.7
Errinundra	-3.2	-3.5	-4.3
Southern N.S.W.	-3.3	-3.6	-4.5
Species mean ± S.D.	-3.4±0.2	-3.6±0.2	-4.5±0.3
Stem damage			
Rubicon	-3.8	-3.8	-4.8
Toorongo	-3.5	-3.9	-4.8
Errinundra	-3.2	-3.8	-4.6
Southern N.S.W.	-3.6	-3.9	-4.5
Species mean ± S.D.	-3.5±0.3	-3.8±0.1	-4.7±0.4

A, frost temperatures -3.4, -3.6 and -3.8°C

B, frost temperatures -3.3, -3.6 and -4.0°C

C, frost temperatures -4.2, -4.7 and -5.3°C

CHAPTER 6

GENETIC AND SEASONAL ASPECTS OF VARIATION IN FROST RESISTANCE IN *E.NITENS*

6.1 Introduction

E.nitens, shining gum, is a fast growing species with a discontinuous distribution in the mountain ranges of New South Wales and Victoria (see Section 3.2 and Figure 3.1). Whilst the species has been harvested in all its regions of occurrence, it is very much a minor species except for the Errinundra and Toorongo provenances. In contrast, *E.nitens* is becoming an increasingly important plantation species for pulpwood production in Australia. In Tasmania, it is the preferred species in new eucalypt plantations, largely due to its rapid early growth over a range of sites and a high level of frost resistance relative to that of other fast growing *Eucalyptus* species (Tibbits 1986). Interest in *E.nitens* is also being shown overseas, as it is generally regarded as one of the more promising *Eucalyptus* species for cold sites in many overseas countries, e.g. South Africa (Darrow 1984), Zimbabwe (Quaile and Mullin 1983), Spain (Arias 1983), France (Marien and Cauvin 1983), some states of the U.S.A. (Hunt 1983), Argentina (Mendonza and Alliani 1983), and New Zealand (New Zealand Forest Service 1980).

The risk of damage to *E.nitens* plantations in Tasmania may have increased in recent years because more plantings have been on colder sites. Indeed, some new plantings in north-west Tasmania suffered considerable frost damage following severe winter frosts in June 1983 (see section 3.5). However, there is only a small amount of information on frost resistance within *E.nitens* (see section 3.5). The most frost resistant provenances of *E.nitens* need to be identified because plantation programmes with *E.nitens* generally use seed from preferred provenances (see section 3.5).

Studies with other *Eucalyptus* species have shown considerable variation in frost resistance with provenance, and particularly altitude (see section 2.3.2). There is likely to be considerable variation in frost resistance within *E.nitens*, since it naturally occurs over a wide latitudinal and altitudinal range. Most other studies on frost resistance within eucalypt species have examined variation at the provenance level only, rather than at the family or individual levels, and few have quantified the differences. The objective of this Chapter was to quantitatively evaluate the variation in frost resistance within *E.nitens* from a large sample covering the natural range of the species. The study was designed to examine the frost resistance of both potted and planted seedlings and at a range of levels of hardiness. In this

study, patterns of variation were examined at the provenance, family and individual levels.

6.2 Materials and methods

Four major experiments were undertaken. The first and second examined the frost resistance of a large number of families subjected to artificial frosting, using unhardened and hardened seedlings respectively. The third evaluated seasonal patterns of frost resistance in a small number of families in a young plantation. The fourth was a pair of field trials, containing a large number of families, that were planted on sites where natural frost conditions were likely to provide a range of damage.

6.2.1 Selection of genetic base

A total of 101 seedlots of *E.nitens* were selected to cover the natural range of the species. All but two of the seedlots were collected from single open-pollinated mother trees, the other two being bulked collections from the Northern N.S.W. provenance, comprising 11 and 12 open-pollinated trees respectively. The term family will be used when referring to seedlings and trees from a single seedlot, though it is not strictly correct in two of the cases. Approximately equal numbers of families were chosen from each of the six provenances as defined by Pederick (1979) (see Table 6.1). Details of each family are given in Appendix A.

All 101 families were used in frosting of unhardened seedlings (Experiment 1) and establishment of field trials (Experiment 4). For Rubicon, Toorongo, and Southern N.S.W. provenances, where the range in altitude of available families covered 400m or more, four altitudinal ranges were sampled. In each of the four ranges (viz., the very low, low, intermediate and high altitudinal range) there were five families, except for the very-low range of the Toorongo provenance where only one family was available (Table 6.1). Within the Errinundra provenance three altitudinal ranges (low, intermediate and high) were used with five families in each. In the Macalister provenance fifteen families were unequally sampled from the high, intermediate and low altitudinal ranges. The Northern N.S.W. provenance generally encompassed a smaller altitudinal range (200 m) and the grouping of seedlots by altitude was not applied, although of the 15 families sampled, 13 were from 1500m (± 20m) and two from 1290m (± 10m). On average for all provenances, the families within each altitudinal range varied in altitude by 40 m , whilst the distance between adjoining ranges was 120 m.

A sub-sample of 36 families was selected for artificial frosting of potted seedlings hardened outdoors (Experiment 2, Table 6.1). Six families from each of the six provenances were selected, with three families belonging to high altitudinal ranges and three belonging to low (intermediate for Macalister) altitudinal ranges {i.e. there was about 320 m difference in the mean altitudes of the two groups of three families (120 m for Macalister)}. For the Northern N.S.W. provenances, three families were chosen from each of two

geographically separate populations Barrington Tops and New England National Park.

Eight families were selected to cover the geographic range of the species (from 50 families represented at a three-year-old progeny trial) for evaluation of seasonal patterns in frost resistance using leaf discs (Table 6.1). Of these eight families, seven were from the subsample of 36 families. Two families, representing relatively high and low altitudes, were selected from Rubicon and Southern N.S.W. provenances and two families representing the two populations were selected from the Northern N.S.W. provenance. The remaining two families were from a high altitude Errinundra provenance and a low altitude Toorongo provenance with "early-adult form".

6.2.2 Artificial frosting of unhardened seedlings (Experiment 1)

Seedlings from all 101 families were grown in polythene pots, one per pot, in a heated glasshouse (see Chapter 4). Mean seedling height was approximately 29 cm, but there were significant differences ($P<0.001$) in the mean number of leaf pairs of the six provenances (Table 6.2) with the Errinundra and Southern N.S.W. provenances having less than the other provenances. The seedlings were sorted into groups of about 24 seedlings. Where possible, each group of seedlings comprised four seedlings from each of the six provenances. One week before frosting the groups of seedlings were transferred from the glasshouse to a growth cabinet with a 10 h photoperiod of mixed fluorescent and incandescent source (60 μmol s^{-1} m^{-2}) and 12°C day/8°C night (the day temperature remained operative for the first hour of darkness).

On the day of frosting seedlings were sorted into four blocks, each containing one seedling from the six provenances. The four blocks were placed in the four quarters of a white plastic box (30 cm by 40 cm by 12 cm high) with seedling position being randomised within blocks. Some seedlings did not stand erect, particularly those from the Southern N.S.W. provenance, and these were supported by staking. The seedlings were frosted and assessed for leaf damage as detailed in Chapter 4. At the same time, the height of the stem killed (this was usually to a node) was measured from both the apex, giving the length of stem killed (LK) and the base, giving the height of stem alive (HA). The total number of nodes killed (NK) and alive (NA) were counted (cotyledons scored as node 0). LK,HA,NK and NA were reassessed after a further four to six weeks and were found to have changed significantly ($P<0.01$) from the first assessment.

Table 6.1 The numbers of families and their mean altitudes for all six *E.nitens* provenances used in artificial frosting studies and field trials.

Population†	Number of families ; mean altitude (m) by provenance					
	Rubicon	Toorongo	Macalister	Erinundra	S N.S.W.	N N.S.W.
Frosting unhardened seedlings (Experiment 1), and field plantings (Experiment 4)						
High	5; 1160	5; 1135	5; 1250	5; 1090*	5; 1380	13; 1480
Intermediate	5; 1000	5; 1040	9; 1130	5; 940*	5; 1200	
Low	5; 935	5; 850**	1; 860	5; 800*	5; 1070	2; 1290
Very-low	5; 610	1; 760*			5; 920	
Frosting leaf discs from seedlings hardened outside (Experiment 2)						
High	3; 1160	3; 1135	3; 1250	3; 1090*	3; 1380	3; 1480
Low	3; 935	3; 850**	3; 860	3; 800*	3; 1070	3; 1410
Frosting leaf discs from a three-year-old progeny trial (Experiment 3)						
High	1; 1160			1; 11201*	1; 1220	1; 1480
Low	1; 940	1; 860*			1; 940	1; 1480

†, according to an altitudinal range (high, intermediate, low or very-low)
*, all families having "early-adult form"
**, one of the families having the "early-adult form"

Table 6.2. Seedling height and number of leaves expanded (>50% length) for the six *E.nitens* provenances used in Experiment 1 (seedlings frosted to -3.2°C).

Provenance	Seedlings	Height (cm) Mean	S.E.	Leaves expanded Mean	S.E.
Rubicon	30	28.8	0.6	11.6	0.2
Toorongo	32	28.9	0.5	11.7	0.2
Macalister	32	29.2	0.5	10.5	0.4
Errinundra	26	28.6	0.5	9.5	0.3
Southern N.S.W.	40	28.7	0.3	9.8	0.2
Northern N.S.W.	29	28.6	0.2	11.7	0.2
Species overall	189	28.6	0.2	10.8	0.1

6.2.3 Artificial frosting of seedlings hardened outside (Experiment 2)

In February 1985, when the seedlings were approximately four months old and 20 cm tall, they were transferred from a glasshouse and placed outside under natural Hobart conditions, without shade but watered regularly. They were arranged in a randomised block design with each block containing 12 seedlings, one seedling from each of the two populations or altitudinal ranges for all six provenances (Table 6.1).

Frost resistance was evaluated in July 1985 using leaf discs, as sample frostings indicated that seedlings were most likely at maximum hardiness. Temperature records from a thermohygrograph located nearby indicated that for the seven week period before frosting the minimum temperature was 4°C or below on 44 of the 49 nights. From May to July approximately seven frosts occurred.

At the time of sampling, one disc was taken for frosting from each seedling at the second or third most recently expanded leaf pair. On average, this corresponded to the ninth to eleventh node. Seedling height was generally 40 cm although the Errinundra and Southern New South Wales provenances were generally 15 cm taller, though they had the same number of nodes. Discs were sampled from three seedlings from each of the 36 families and the 108 discs frosted to one of three temperatures. This was subsequently repeated for other temperatures. A second group of three seedlings from the 36 families were then treated in a similar way. Discs from a seventh seedling from each of the families were then frosted.

6.2.4 Seasonal variation in frost resistance of plantation trees (Experiment 3)

The trees chosen were part of a progeny trial established in October 1982 and were located in the Huntsman Valley at the foothills of the Western Tiers, near Deloraine, Tasmania (Figure 3.1). The site was level at an altitude of 420 m with a rocky soil derived from alluvial outwash of dolerite parent material. Prior to clearing the site was eucalypt forest with *E.ovata* Labill. as the dominant species. For each family, eight trees were sampled from single plots (three rows by three columns). The eight plots sampled were confined to an area of 0.4 ha. Standardized maximum/minimum thermometers were set up at breast height on star-pickets in the four corners and mid-point of the 0.4 ha area. Maxima and minima temperatures were read approximately every three weeks.

On each tree three branches at breast height, covering a range of aspects, were selected and one fully expanded leaf was carefully cut from each branch. All families retained juvenile foliage except for the Errinundra and Toorongo families ("early-adult form"). The leaves were immediately placed in sealed plastic bags and kept cool in an insulated container and subsequently in a cold room at 5°C until frosting the next day. Sampling took place from the same branches in March, May, July, September and November 1985 and February1986. In August 1985 a subsample was made of the trees

from the low altitude Rubicon and Southern N.S.W. families only.

Frosting was carried out as for seedlings hardened outside (Experiment 2) except that two discs (6 mm diameter) were taken from each of three leaves, one each side of the mid-rib, and the six discs for each tree placed in a vial for frosting. At each sampling, discs were usually frosted to one of three temperatures set 1.0 to 1.5°C apart.

The rationale for the use of a number of smaller discs here, as developed by Hallam (1986), is as follows:-

1) a single larger disc per vial from two or three leaves would have exceeded the acceptable working capacity of the freezing chamber; and

2) it was desirable to assess frost resistance of the trees using a number of leaves around the tree. The crown spread of each tree, and hence the likely variation in micro-environment it is subjected to, was much larger than that of any other seedlings used in this study.

6.2.5 Field trials (Experiment 4)

Seedlings for the field trials were grown in spring of 1984 at the Associated Forest Holdings Pty. Ltd. (A.F.H.) nursery at Ridgley, Tasmania. Seed was sown onto fine granitic sand in punnets and germinated in a hot-house. Approximately two weeks after germination (before the first leaf pair had expanded) the germinants were transplanted into paper pots (3.0 cm diameter and 7.5 cm deep). A basalt soil was used with a slow release NPK fertiliser, at the rate of 1.0 g seedling^{-1}. Approximately 140 seedlings of each family were transplanted.

There were two field trial locations; Hampshire, altitude 400 m, and Racecourse Plains, altitude 650 m, both on A.F.H. freehold land south of Burnie, Tasmania (Figure 3.1). In May 1984 weather stations were established at both sites. The trial at Hampshire was within a routine plantation, whilst the trial at Racecourse Plains was in an area specifically prepared for this and other experiments. Both sites were non-forested with a heavy grass sward. The Hampshire site was once forested but cleared many decades earlier, whilst the Racecourse Plains site was not forested at the time of European settlement. At the Hampshire site planting lines were mound ploughed during Autumn 1984. Two weeks before planting the experimental area was broadcast sprayed with a knockdown herbicide, Roundup (Monsanto Aust. Ltd.), at a rate of 1.0 litre active ingredient (a.i.) per hectare. In Autumn 1985, four months after planting, planting lines and seedlings were oversprayed with a pre-emergent herbicide, Atrazine (Monsanto Aust. Ltd.), at a rate of 12 litres (a.i.) per hectare. There were no visibly deleterious effects of the overspraying on seedling health. The Racecourse site was disc ploughed twice during late Summer 1983/4 and planting lines were mound ploughed in Autumn 1984. The area was broadcast sprayed with the pre-emergent herbicide (as above) in Autumn 1984 and again in Autumn 1985.

The trials were established using a randomised complete block with 11 by 11 single tree plots (3 m square spacing), replicated 24 times. Of the 121 single trees, 97 were

E.nitens families and 24 were two seedlots of each of 12 other *Eucalyptus* species (see Appendix B). There were four fewer *E.nitens* families than the 101 used in the artificial frosting studies with unhardened whole seedlings (Experiment 1), because each of the five families from the very-low altitude Rubicon population yielded insufficient seedlings and these were combined into a single seedlot (Table 5.1).

Unseasonally cool weather prevailed in Spring 1984 and seedlings were not ready to plant until mid-December (when most seedlings were ≥10 cm tall). Planting commenced mid-December 1984 but only 12 replications at Racecourse were able to be planted because of heavy rainfall. Planting was not able to recommence until early January 1985. The remaining 36 replications were planted within four days. By this time the soil had dried out considerably despite the heavy rain only two weeks earlier. As a result, there was about 15% mortality at each site (Table 7.1). Seedlings were fertilised in April/May 1985 with *c.* 60 g super-phosphate placed just under the soil surface and 20 cm away from the seedling base.

Survival was assessed in May 1985 so that seedlings which had died from causes other than frost could be accounted for before damaging frosts occurred. In July 1985 frost damage was visually assessed on a scale of 0-5 (after Menzies *et al.* 1981), viz.,

0, no damage to foliage or stem

1, up to 10 % foliage damage (usually no stem damage)

2, 11 % to 29 % foliage damage (usually no stem damage)

3, 30 % to 69 % foliage damage (variable levels of stem damage)

4, 70 % to ca.90 % foliage damage (often severe stem damage)

5, 100 % foliage damage (stem usually killed to ground level)

6.2.6 Statistical analysis

Percentage leaf damage scores were not normally distributed and analysis was undertaken using non-parametric tests. The scores were arc-sin transformed and Kruskal-Wallis one-way analysis of variance used to test amongst provenances and one-tailed Mann-Whitney U-tests were used to test differences between pairs of provenances or populations (Anon. 1986). Stem damage and frost temperatures causing 50% loss of cellular electrolytes were approximately normally distributed and analysis amongst and within provenances were carried out using analysis of variance. Visual assessments of frost damage were not normally distributed, the frequencies of plants in the damage classes were analysed using the G-test of goodness of fit (Sokal and Rohlf 1981).

6.3 Results

6.3.1 Genetic variation of unhardened seedlings (Experiment 1)

A frost temperature of -2.9°C for 90 min was not sufficient to cause visual damage to seedling leaves or stems whilst more than 90% of leaf area and stem height were killed by a frost only 1.2°C colder (-4.1°C, Table 6.3). Stems suffered slightly less proportionate damage than leaves at equivalent temperatures. Over the range of temperatures -3.2 to -3.8°C there were highly significant ($P<0.001$) differences in the mean damage to leaf area and stem height amongst the provenances. The least frost resistant were the Errinundra and the Southern N.S.W. provenances. The other four provenances suffered less damage and were generally not significantly different from each other. Seedlings from the Errinundra and Southern N.S.W. provenances have reduced ability for stem recovery compared with other provenances because of the more extensive stem damage (Table 6.3) and the fact that they had fewer nodes in a given height (Table 6.2). The most severe frost of -4.1°C was so damaging that overall no major differences occurred amongst the provenances ($P>0.01$). Within each provenance and at each temperature, there were no significant differences in leaf or stem damage amongst altitudinal groups or between populations (i.e. Barrington Tops c.f. New England National Park in the Northern N.S.W. provenance). Family differences were not analysed because of the low numbers of seedlings from each family which were frosted to each temperature. The mean temperature resulting in 50% leaf damage, which has been used as an index of damage (see Harwood 1980), was about -3.2°C for unhardened *E.nitens* seedlings (Table 6.3).

The percentage of seedlings killed by the frostings also showed clear and consistent differences amongst the provenances, with the Errinundra and Southern N.S.W. provenances characterised by higher mortality than the other provenances (Figure 6.2). There was some indication that the Northern N.S.W. and Macalister provenances were more resistant than the Rubicon and Toorongo provenances at temperatures of -3.5 and -3.8°C.

6.3.2 Genetic variation of hardened seedlings (Experiment 2)

The seedlings hardened outside had an overall mean frost resistance of -5.4°C (Table 6.4a), indicating that they had hardened about 2.2°C from the unhardened state (Table 6.3). Partitioning the variation in frost resistance of the 252 seedlings showed that provenances accounted for 75% of the variation (Table 6.5a). The variation amongst seedlings (13%) was high compared with that between populations (8%), amongst families within populations (3%), or amongst families within provenances *per se* (8+3%).

Table 6.3 Variation in relative frost resistance of unhardened *E.nitens* seedlings. Given are the number of seedlings frosted, and mean (S.E.) increase in stem damage from two weeks to two months (LK2-LK1), and leaf and stem damage for all six *E.nitens* provenances (n>20) used in artificial frosting studies. Significance levels amongst provenances are:- not significant (NS), P<0.05 (*), P<0.01 (**), and P<0.001 (***).

	Frost temperature (°C)				
	-2.9	-3.2	-3.5	-3.8	-4.1
Number seedlings	87	189	166	160	135
LK2-LK1 (cm)	0	3.3 (0.4)	3.7 (0.5)	4.4 (0.5)	3.9 (0.5)
(a) Mean Leaf Damage (%)					
Significance	NS	***	**	***	*
Provenance					
Northern N.S.W.	0	35 (7.4)	66 (8.4)	74 (8.1)	96 (2.0)
Rubicon	0	39 (7.1)	74 (7.0)	96 (2.0)	94 (2.2)
Toorongo	0	49 (6.9)	71 (7.9)	89 (4.4)	85 (7.8)
Macalister	0	54 (6.8)	69 (6.6)	89 (3.1)	93 (4.5)
Errinundra	0	54 (9.1)	86 (7.5)	100 (0.0)	94 (6.2)
Southern N.S.W.	0	81 (4.8)	88 (4.2)	98 (1.2)	100 (0.0)
Species Mean	0	54 (3.0)	75 (2.8)	91 (1.4)	94 (2.0)
(b)					
Mean Length of Stem Damage (cm)					
Significance	NS	***	**	***	NS
Provenance					
Northern N.S.W.	0	7.4 (2.2)	13.6 (2.5)	15.8 (2.9)	23.7 (1.9)
Rubicon	0	7.5 (1.9)	18.1 (2.2)	23.3 (1.9)	25.5 (1.7)
Toorongo	0	9.4 (2.2)	16.3 (2.6)	21.3 (2.3)	24.7 (2.3)
Macalister	0	10.7 (2.2)	16.1 (2.2)	20.2 (2.5)	26.9 (2.7)
Errinundra	0	14.6 (2.7)	23.3 (2.4)	31.9 (1.1)	28.8 (2.0)
Southern N.S.W.	0	20.6 (1.7)	24.0 (1.5)	26.2 (0.9)	29.2 (0.6)
Total Height (cm)	27.7	28.6 (0.9)	28.4 (0.9)	29.6 (0.8)	29.6 (0.6)

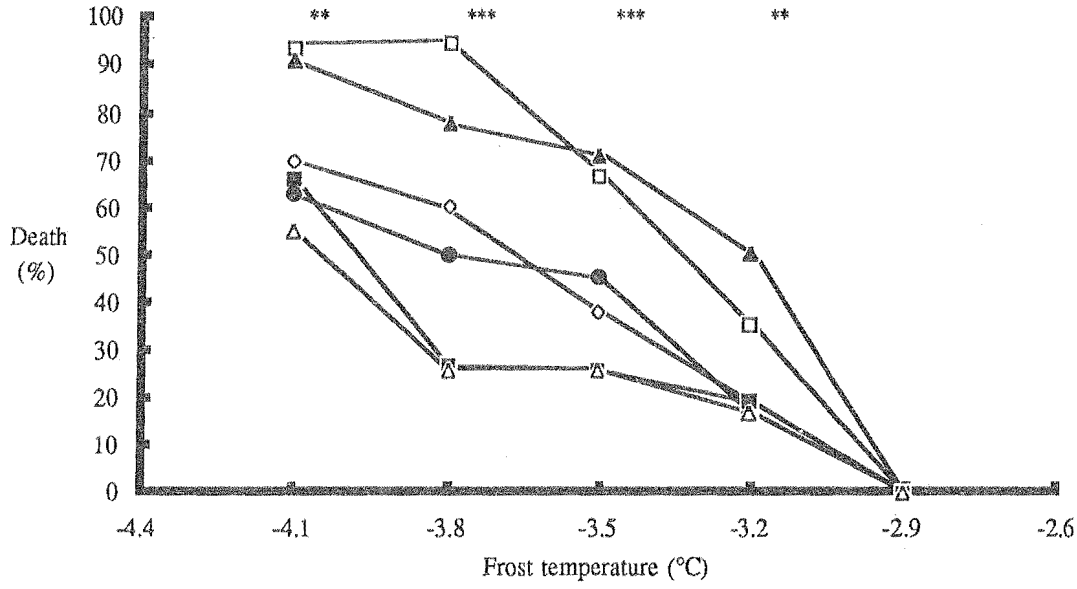


Figure 6.1. Relationships between seedling death (%) and frost temperature for unhardened *E.nitens* seedlings. Provenances are, Rubicon (●), Toorongo (◇), Macalister (■), Errinundra (□), Southern N.S.W. (▲) and Northern N.S.W. (△). Levels of significance amongst provenances are, ** (P<0.01) and *** (P<0.001).

There were highly significant differences amongst the provenances (P<0.01), with the Errinundra and Southern N.S.W. provenances again having the lowest levels of frost resistance (Table 6.4a). The other four provenances were not significantly different from one another and the Toorongo provenance was not significantly different in its frost resistance from any of the other provenances. Differences in T50 (temperature of 50% leakage cellular electrolytes) between altitudinal ranges (i.e. populations) were only significant (P<0.01) within the Southern N.S.W. provenance, where the families from the low altitudinal range were on average 0.6°C less frost resistant than those from the high altitudinal range. There were significant differences in levels of frost resistance amongst families within each of the provenances except for the Northern N.S.W. provenance (Table 6.4a). Although the maximum difference between provenances was only 0.7°C, the maximum difference between families was 1.6°C.

Table 6.4 Variation in relative frost resistance of hardened *E.nitens*. Frost resistance assessed by, (a) frost temperatures resulting in 50% leakage of cellular electrolytes (°C) for seedlings hardened outdoors, and (b) visual scores of damage following natural frosts at the Racecourse Plains trial. Provenances are, Rubicon (R), Toorongo (T), Macalister (M), Errinundra (E), Southern N.S.W. (S), and Northern N.S.W. (N). Significance levels amongst provenances, populations and families are, not significant (NS), P<0.05 (*), P<0.01 (**), and P<0.001(***). Mean values, for provenances joined by lines are not significantly different (P>0.05). Mean values for altitudinal populations and families with different letters are significantly different (P<0.01).

Level of comparison	Provenance N	R	M	T	E	S	Overall
a.							
Significance							
Between pop.*	NS	NS	NS	NS	NS	**	*
Within pop.	NS	**	**	**	**	NS	***
Altitudinal pop.							
Low	-5.6†	-5.6	-5.7	-5.2	-4.9	-4.7	-5.3
High	-5.6††	-5.8	-5.6	-5.4	-5.3	-5.3	-5.5
Mean	-5.6	-5.7	-5.6	-5.3	-5.1	-5.0	-5.4
Family extremes							
Maximum**	-5.9ab	-6.2a	-5.8abc	-6.0ab	-5.8abc	-5.5bc	-
Minimum	-5.4bcd	-5.5bc	-5.0bd	-5.2c	-4.6d	-4.7d	-
b.							
Significance							
Between pop.	***	*	*	NS	**	**	-
Within pop.	***	**	**	**	**	**	***
Altitudinal pop.							
Very-low	-	2.2c	1.9b	2.5c	-	3.1c	2.9
Low	2.3b	1.4ab	-	1.8ab	3.2b	2.8b	2.6
Intermediate	-	1.5b	1.8b	1.9b	2.2a	2.4b	2.0
High	1.2a	1.1a	1.0a	1.4a	2.2a	1.7a	1.6
Mean	1.4	1.4	1.5	1.7	2.5	2.5	1.8
Family extremes							
Maximum[z]	0.6a	0.7ab	0.7ab	1.3ab	1.5bc	0.8ab	-
Minimum	2.2cd	2.8de	2.4d	3.8f	3.4ef	2.4d	-

*, altitude population (see Table 6.1); **, most frost resistant

†, New England population ; †† Barrington Tops (Northern N.S.W. provenance)

Table 6.5 Analysis of variance tables for estimation of variance components within *E.nitens*. For (a) frost temperatures resulting in 50% leakage of cellular electrolytes (°C) for seedlings hardened outdoors, and (b) visual assessment of damage following natural frosts at the Racecourse site.

Source	D.F.	S.S.	M.S.	Expected M.S.	Solution	%
a.						
Prov.*	5	18.353	3.6706	$\sigma^2_s+7\sigma^2_f+3\sigma^2_a+2\sigma^2_p$	$\sigma^2_p=1.3122$	75
Alt.†	6	6.278	1.0463	$\sigma^2_s+7\sigma^2_f+3\sigma^2_a$	$\sigma^2_a=0.1403$	8
Fam.**	24	15.011	0.6255	$\sigma^2_s+7\sigma^2_f$	$\sigma^2_f= 0.0563$	3
Seed.††	212	49.040	0.2313	σ^2_s	$\sigma^2_s=0.2313$	13
Total	247	88.682				
b.						
Prov.	5	451.48	90.297	$\sigma^2_s+3\sigma^2_b+8\sigma^2_f+15\sigma^2_p$	$\sigma^2_p=5.503$	73
Fam.	84	651.58	7.757	$\sigma^2_s+3\sigma^2_b+8\sigma^2_f$	$\sigma^2_f=0.791$	10.5
Block	625	892.50	1.428	$\sigma^2_s+3\sigma^2_b$	$\sigma^2_b=0.107$	1.5
Seed.	1105	1222.3	1.106	σ^2_s	$\sigma^2_s=1.106$	15
Total	1819	3217.9				

*, provenance

†, altitudinal population

**, family

††, seedling

Variation (%) of "seedlings-within-provenances" = Pop. + Fam. + Seed., or = Fam. + Seed.

6.3.3 Seasonal variation (Experiment 3)

There were large changes in frost resistance throughout the year in each of the eight families sampled from the progeny trial. Near the end of summer, i.e. March 1985 and February 1986, all families were at their lowest (least negative) level of frost resistance. The critical temperatures in summer causing 50% loss of cellular electrolytes from leaf discs (Table 6.6) and those causing 50% leaf damage to unhardened potted seedlings (Table 6.3) compare favourably ,viz., -3.4 and -3.2°C respectively, indicating the plantation trees were at about their minimum level of hardiness. In the middle of winter, excised leaf tissue had hardened to about -10.0°C (Table 6.6), i.e. almost 5°C more resistant than the seedlings left to harden outside in Hobart (Table 6.4a).

There were significant differences ($P<0.01$) in T50 values amongst the families at all times, but the range was less in mid-summer than in mid-winter, viz., 0.8 and 2.3°C respectively. The low altitude Southern N.S.W. family (S17) was the least frost resistant family at five of the six dates of sampling, whilst the low altitude Rubicon family (R14) was always one of the most frost resistant families, and attained the maximum level of hardiness. Although there were only one or two families per provenance the relative ranking of provenances agreed with that found from artificial frosting studies of potted seedlings (Tables 6.3 and 6.4a). The change in hardiness, (i.e. the change in T50 from one date to another) also varied significantly amongst the families as they hardened and dehardened (even for the late dehardening phase over summer, when on average the level of frost resistance changed by less than 1.0°C) (Table 6.6). The mean maximum rates of hardening during winter and dehardening over spring of 0.4°C $week^{-1}$ are quite small but in good agreement with that found under controlled environmental conditions for *E.nitens* (Chapter 5).

An additional aspect of the frost resistance of the trees that varied over the year was the increase in level of damage with increasing frost temperature, i.e. the slope of the relationship between relative conductivity and frost temperature. As seedlings hardened from summer to winter, the slope halved, from 30% $°C^{-1}$ or above to 15% $°C^{-1}$, indicating that the temperature range over which damage progresses from very slight to severe was broader in winter than in summer (Figure 6.3a). Generally, the differences of these mean slopes amongst families were not significant. The reason for the significant difference in mean slopes between March 1985 and February 1986 is not known.

A frost of ca. -4.5°C in late April resulted in damage to *c.* 25% of *E.fastigata* foliage (included in the trial area) and 10-15% foliage damage to the Toorongo family with the "early-adult-form" (T11), in a separate part of the trial. No damage was observed to any other families of *E.nitens* in any other part of the trial, except minor bud death.

Table 6.6 Variation in relative frost resistance of three-year-old plantation-grown *E.nitens*, from March 1985 to February 1986. Given are, (a) frost temperatures resulting in 50% leakage of cellular electrolytes (T50), (b) changes in T50 between consecutive dates, and (c) the slope of plots of relative conductivity against frost temperature. Families are:- low elevation Rubicon (R14) ; high elevation Rubicon (R03); low elevation "early-adult form" Toorongo (T11); high elevation Errinundra (EH); low elevation Southern N.S.W. (S17); high elevation Southern N.S.W. (SH); New England National Park, Northern N.S.W. (N13), and ; Barrington Tops Northern N.S.W. (N11). Changes in hardiness are, March-May (A), May-July (B), July-Sept.(C), Sept.-Nov.(D) and Nov.-Feb.(E). Least significant differences (L.S.D.) between families at a given sampling date are indicated ($P<0.01$).

	Family†								
Time (L.S.D.)	R14	R03	T11	EH	S17	SH	N13	N11	Mean
(a) T50 (°C)									
March (0.6)	-3.9	-3.6	-3.2	-3.1	-3.7	-3.3	-3.9	-3.4	-3.5
May (0.7)	-5.7	-5.8	-4.7	-4.8	-4.6	-5.7	-5.7	-6.3	-5.4
July (0.9)	-10.7	-10.1	-10.2	-9.7	-8.4	-10.0	-10.2	-10.2	-10.0
Sept. (0.9)	-6.4	-5.9	-6.8	-6.5	-5.1	-6.2	-6.0	-6.4	-6.2
Nov. (0.6)	-4.8	-4.2	-5.1	-4.1	-3.6	-4.2	-4.0	-4.1	-4.2
Feb. (0.6)	-3.9	-3.2	-3.3	-3.2	-3.0	-3.4	-3.1	-3.2	-3.3
(b) Change in T50 (°C;°C week^{-1})									
A (0.8)	1.8	2.2	1.5	1.6	0.9	2.4	1.8	2.9	1.9;0.2
B (0.7)	5.0	4.2	5.5	4.7	4.0	4.2	4.7	4.0	4.6;0.4
C (0.9)	-4.3	-4.1	-3.4	-3.1	-3.5	-3.8	-4.4	-3.9	-3.8;0.4
D (0.9)	-1.7	-1.7	-1.8	-2.4	-1.5	-2.0	-2.0	-2.3	-1.9;0.2
E (0.6)	-0.8	-1.0	-1.8	-0.8	-0.6	-0.8	-0.9	-0.9	-0.9;0.1
(c) Slope of relative conductivity against frost temperature (% °C^{-1})									
Mean (NS)	21	28	28	32	28	29	27	28	27

†, as in Appendix A (except for EH and SH which were not in the 101 families selected)

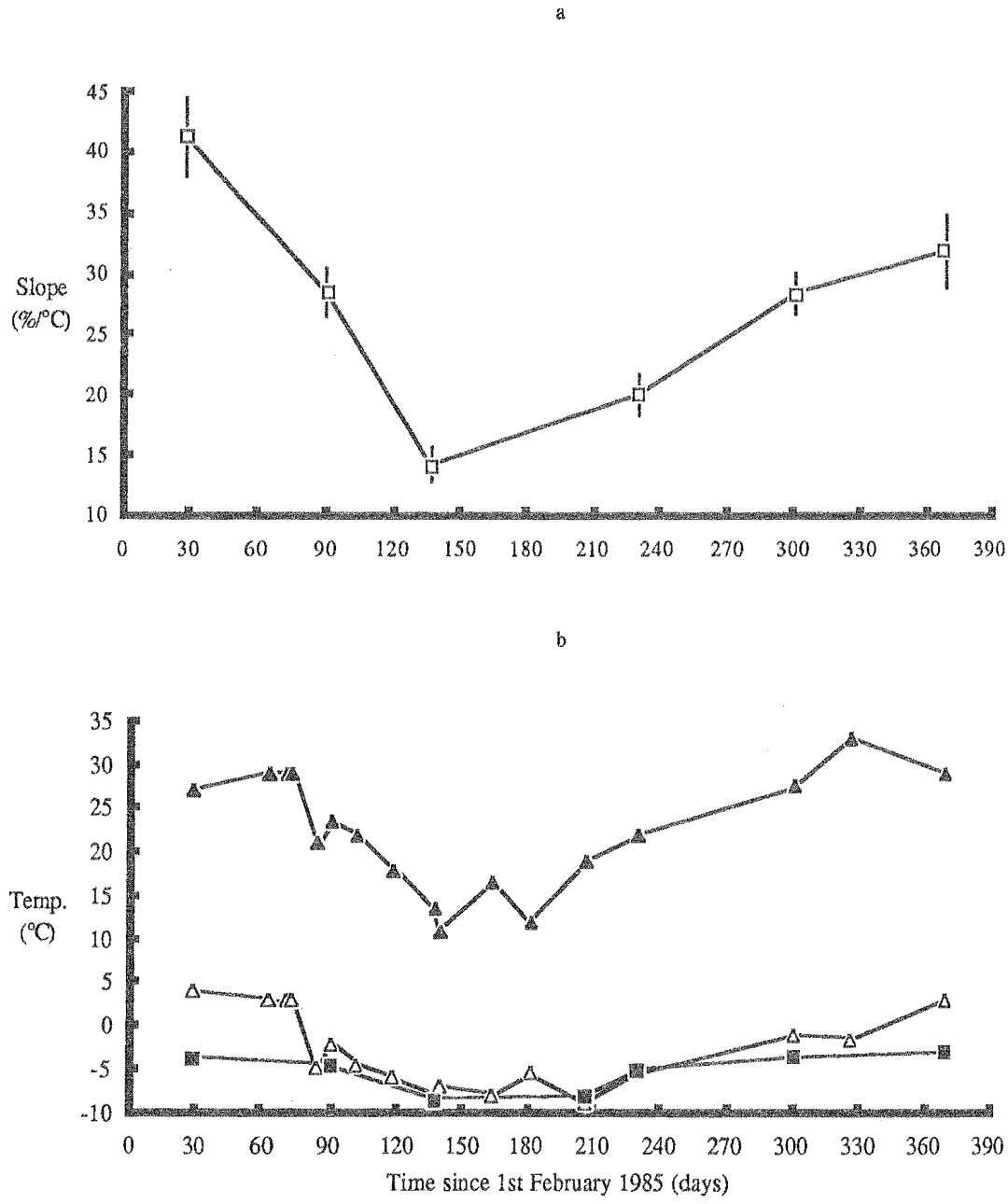


Figure 6.2 Seasonal trends in relative frost resistance and ambient temperatures at the Huntsman progeny trial. Given are, (a) the mean slopes (and 95% confidence intervals) of the relationships between relative conductivity (of the bathing medium containing frosted leaf discs) and frost temperature, and (b) mean maximum (▲) and minimum (△) temperatures and T50 (■) for the low elevation Southern N.S.W. family (see Table 6.6).

6.3.4 Field trials (Experiment 4)

Thermohygrograph and maximum/minimum temperature records from the two sites revealed that both sites were subjected to frequent frosts (see Figure 7.1), with minima of -7 and -10°C during July 1985, at Hampshire and Racecourse respectively.

At the Hampshire site, overall levels of frost damage were very low at 0.4, i.e. less than 10% foliage damage. There were no significant differences amongst provenances or altitudinal populations. In contrast, the average level of damage at Racecourse Plains was 1.8, i.e. almost 30% foliage damage. Though this average level of damage was still quite low, there were highly significant differences amongst the provenances (Table 6.4b). Wilcox (1982c) found that similar low levels of damage to *E.fastigata* also resulted in significant differences amongst provenances. Overall, the Southern N.S.W. and Errinundra provenances were characterised by significantly higher levels of damage (i.e. 2.5 or almost 50% foliage damage, $P<0.01$) than the other four provenances (mean damage scores between 1.4 and 1.7).

Within each of the five provenances, where families were usually subdivided into sets of usually five families at a common altitudinal range (Table 6.1), there were significant differences in damage levels amongst the altitudinal ranges. Generally speaking, the trend was for decreasing damage (increasing frost resistance) with increasing altitude (Table 6.4b). There were also highly significant differences in the frost damage to families within provenances (Table 6.4b). Overall, the differences between the most frost resistant family from each provenance were not significant ($P>0.01$). In contrast, the families with the most damage from the more frost resistant provenances were as frost resistant as the mean of the less frost resistant provenances (Table 6.4b).

A variance component analysis of 90 families grouped into the six provenances (15 families per provenance which excluded families from very-low altitude populations, Table 6.1) showed that the "provenances" components were three times larger than the "seedlings-within-provenances" components (Table 6.5b). In addition, the component for "seedlings-within-families" was larger than the component for "families-within-provenances". This closely agrees with the results from seedlings hardened outdoors (Table 6.5a).

6.4 Discussion

These studies were primarily designed to identify those provenances of *E.nitens* with the highest level of frost resistance. The findings from the artificial frosting experiments and the natural frost damage to the field trials consistently identify two groups of provenances with differing levels of frost resistance. The Western provenance, from the Central Highlands of Victoria, and the provenance in Northern N.S.W. show superior

hardiness to the Southern N.S.W. and Errinundra provenances. This was demonstrated for plants that were basically unhardened (Table 6.3), partially hardened (Table 6.4a) and at near maximum hardiness (Table 6.4b). This is of considerable practical significance as damaging frosts can occur not only in winter, but also in both autumn and spring, when plants are at lower levels of hardiness (see Davidson and Reid 1987). Rook *et al.* (1980) found only a slight provenance by season interaction in the relative frost resistance of *E.regnans*, which was of little practical application. However, as plantation trees dehardened the relative ranking of families changes (Table 6.6). This family by "seasonal level of hardiness" interaction was highly significant ($P<0.001$). Hence, although the data are for only one season, they indicate that hardiness by seasonal interactions are important, at least at the family level.

The range in frost resistance within *E.nitens*, as assessed by temperatures causing about 50% leakage of cellular electrolytes, varied from levels of about -3.0°C in mid-summer to -10.0°C in mid-winter (Table 6.6). Even in an unhardened condition (Table 6.3), it was possible to separate the two groupings, at frost temperatures of between -3.2 and -3.8°C. However, the overall level of frost resistance was low, with the critical frost temperature (50% leaf damage) separating the most and least hardy provenances only *c.* 0.3°C (Table 6.3). Although the relative differences in frost resistance between provenances increased as plants hardened, e.g. 0.7°C (Table 6.4a), it was when they were fully hardened that the differences were most clearly expressed. For instance, individual families were found to differ by as much as 2.3°C (Table 6.6) c.f. 1.6°C when they were partially hardened (Table 6.4a). In a winter hardened condition the full range in frost resistance of the provenances was not assessed (in terms of T50) but the data from Table 6.6 suggests that the least and most frost resistant provenances probably differ by *c.* 1.0°C.

These differences between frost hardy and frost sensitive provenances of *E.nitens* are not as large as those reported for other *Eucalyptus* species, such as *E.regnans* (Rook *et al.* 1980), *E.delegatensis* (Hallam 1986), and *E.fastigata* (Wilcox 1982c). However, this probably only reflects a difference in the definition of a provenance. The six *E.nitens* provenances, as defined by Pederick (1979), generally cover a much broader geographical and altitudinal range than the provenances used in other frost resistance studies. For example, Rook *et al.* (1980) subdivided the *E.regnans* into 38 provenances, most provenances varying little in altitude, and some located only ca. 10km apart. If this definition was used for *E.nitens*, the range in relative frost resistance between the "new provenances" would probably increase to 1.0°C for partially hardened seedlings (Table 6.4a) and *c.* 2.0°C in winter (Tables 6.6).

In many *Eucalyptus* species frost resistance has been shown to increase with the colder conditions of either higher altitudes, e.g. *E.regnans* (Eldridge 1969;Rook *et al.* 1980), *E.urnigera* (Thomas and Barber 1974), *E.pauciflora* (Pryor 1957a), *E.fastigata* (Sherry and Pryor, 1967; Wilcox 1982c), *E.viminalis* (Paton 1972) and *E.delegatensis* (Grose 1960; Hallam, 1986), or frost hollows, e.g. *E.regnans* (Ashton 1958) and *E.pauciflora.* (Harwood 1980). The same has been shown for other forest trees, eg. *Sequoiadendron*

giganteum (Lindley) Buchhloz (Guinon *et al* 1982). The same appears to occur in *E.nitens*, since within provenances those families from higher altitudes were characterised by superior frost resistance, particularly at well developed levels of hardiness (Table 6.4b). There are no instances of families from frost hollows used in this study. Hence, in *E.nitens* there may be greater selection against frost sensitive individuals with the colder conditions at higher altitudes.

The components of variance in frost resistance for "provenances" were about three times larger than components for "seedlings-within-provenance", for seedlings subjected to both artificial and natural frosts (Table 6.5). This agrees with the findings for *E.fastigata* (Wilcox, 1982c). However, the larger component of variation for "seedlings-within-families" than for "families-within-provenance" indicates that there is scope for individual selection in a breeding programme incorporating frost resistance as an important trait. Selection at the individual and family level and controlled breeding for frost resistance, using both interspecific and intraspecific hybrids and vegetative propagation, is underway in France (Potts and Potts 1986).

The maximum rates of hardening and dehardening in planted *E.nitens* of about 0.4°C $week^{-1}$ (Table 6.6) are in good agreement with those shown under controlled environmental conditions (Chapter 5). They also agree with that found for naturally hardening *E.saligna* Sm., *E.regnans* and *E.fastigata* (Menzies *et al.* 1981). These relatively low rates of hardening help explain the severe frost damage to *Eucalyptus* species in many parts of Europe which occur following the onset of sudden frosts, without substantial periods of night temperatures sufficiently low for hardening to take place. Changes in relative frost resistance of the progeny trial (T50 and the slope, Table 6.6) closely followed those of mean minimum site temperatures (Figure 6.3b). Controlled environmental studies have shown that minimum temperatures have the more bearing on frost resistance than maximum temperatures (Chapter 5).

There appear to be different types of hardening and dehardening responses throughout the year for those seven families which attain T50 levels of *c.* -10.0°C in winter (Table 6.6). The two families with the "early-adult-form" are initially slow to harden and remain relatively frost sensitive in autumn. However, by mid-winter the two families with the "early-adult-form" had hardened to similar levels as the other families, except the low altitude Rubicon (R14, more resistant) and Southern N.S.W. (S17, less resistant) families. In contrast, the two families with the "early-adult-form" were slower in their initial stages of dehardening and consequently possessed above average frost resistance in early spring. By summer they had fully dehardened.

The association of a low level of frost resistance with the "early-adult-form" of *E.nitens* in the Errinundra provenance is reinforced by the results of two families from the Toorongo plateau with this form which were included in the field trials. On average, the mean damage to these families at the Racecourse site was 50% greater than that to the provenance as a whole, viz., 2.6 c.f. 1.7. Although these families were from relatively low altitudes, they still suffered significantly more damage ($P<0.01$) than other families collected

from similar altitudes (825 to 850 m). This is supported by the findings of Nixon (1977), where one family from the Toorongo provenance with the "early-adult-form" (from near Christmas Creek) was severely damaged whilst others ("juvenile-persistent form") were not. However, the "early-adult-form" *per se* is not necessarily responsible for reduced levels of frost resistance. Of interest is the poor frost resistance of the Southern N.S.W. provenance. Hence, the two forms suggested by Pederick (1979) may well be an over-simplification of the genetic variation within *E.nitens*. The Southern N.S.W. provenance is characterised by early growth rates and leaf shapes that are intermediate to the "early-adult" and "juvenile-persistent" forms (Pederick 1979). It is also decidedly less glaucous than the more frost resistant provenances (personal observations).

Whilst the frost sensitive Errinundra and Southern N.S.W. provenances are characterised by narrower juvenile leaves of less area (Pederick 1979) than the other provenances, leaf area and/or shape *per se* are not causally related to frost resistance, as evidenced by the separation of provenances using leaf discs of uniform shape and size (Tables 6.4a and 6.6). The Errinundra and Southern N.S.W. provenances are decidedly less glaucous than the other provenances, and this may be linked with frost resistance in some way (Thomas and Barber 1974). Nixon (1977), found a significant negative correlation between winter frost damage and juvenile leaf glaucousness. However, Paton (1981) found no evidence that frost resistance and leaf glaucousness were linked in either segregating progeny of *E.urnigera* or F_2 and backcross progenies between *E.pulverulenta* and *E.grandis*. Similarly, no correlations have been reported in France (Cauvin *et al.* 1987).

No damage was observed in the low altitude Southern N.S.W. family in the progeny trial even though the minimum air temperatures in winter were almost the same as the level of frost resistance assessed (Figure 6.3b). This may be because the frost resistance levels obtained using the leaf disc method are not necessarily absolute and are best used for comparative work. For instance, a slower rate of cooling (*c.* 3°C h^{-1}) will result in a lower T50 value (Chapter 4). The natural rates of cooling to frost temperatures are less than that used here (see Davidson and Reid 1985), and hence absolute frost resistance may be underestimated. The findings show that the leaf disc method of evaluating frost resistance is an effective way of separating provenances and families. Both the leaf disc and whole seedling methods would appear to give similar findings.

CHAPTER 7

FROST RESISTANCE AND EARLY GROWTH OF *E.NITENS* AND 12 OTHER *EUCALYPTUS* SPECIES

7.1 Introduction

In their natural environment eucalypts grow on sites which range from coastal and/or tropical locations, where frosts are absent, to subalpine and/or cool temperate locations, where frosts are frequent (Turnbull and Eldridge 1983). There are correspondingly large differences in frost resistance (Sakai *et al.* 1981) and growth rates (Cotterill *et al.* 1985) within the genus. Most interest in these and other aspects of the eucalypts is shown overseas, where species are planted throughout many countries of the world (Pryor 1978). Indeed, approximately six million hectares of eucalypt plantation have been established overseas (K.G.Eldridge, pers. comm.), compared with less than one percent of that area planted in Australia (Tibbits 1986). Significant proportions of some of the larger overseas plantings are with single preferred species, e.g. there are *c.* three million hectares of *E.grandis* in South American countries (K.G.Eldridge, pers. comm.). However, in many overseas countries the testing of species continues, often involving trials with up to 100 species or large numbers of provenances of selected species, e.g. Brazil (Mendonza and Alliani 1983), South Africa (Nixon 1983), some states of the U.S.A. (Hunt 1983), France (Potts and Potts 1986) and Spain (Arias 1983). In many of these countries, species, provenances and/or families are required that will not only be fast growing but which are also quite frost resistant.

In Australia, as Cotterill *et al.* (1985) point out, testing of eucalypts in plantations has largely "tended to concentrate on comparing provenances of a single species, rather than first determining the relative performance of alternative species", e.g. *E.nitens* (Pederick 1979), *E.regnans* (Griffin *et al.* 1982b), *E.obliqua* (Brown *et al.* 1976), and *E.delegatensis* (Boland and Dunn 1985). Although a number of eucalypt species have recently been tested in the south-east Australian mainland (Cotterill *et al.* 1985) and Tasmania (Tibbits 1986), no evidence in the literature could be found of species trials established in Australia to specifically examine both the relative frost resistance and early growth rate. Ideally, such work should quantify the differences amongst the species. This is particularly relevant to the aspect of frost resistance, where most data are reported simply as relative survival following exposure to freezing temperatures (e.g. Evans 1983) rather than absolute differences between species, i.e. temperatures causing given levels of damage (e.g. Sakai *et al.* 1981; Hallam 1986). Appropriate research into both aspects will not only quantify the fundamental variation in this important genus, but should aid selection and lead to improvement in trees capable of enduring low temperature stress and/or achieving greater biomass production on particular sites.

This chapter reports on the frost resistance of *E.nitens* and 12 other eucalypt species planted on two frost prone sites in north-west Tasmania. *E.nitens* is a preferred plantation species in Tasmania because of rapid early growth over a range of sites and satisfactory frost resistance (Tibbits 1986). It also shows promising growth on the mainland (Cotterill *et al.* 1985). However, its growth on frost prone sites relative to that of a range of other species remains largely unresearched. Species, and provenances within species, were chosen which were likely to represent a range in frost resistance and growth, and where possible were located close to *E.nitens* stands, or could be cross-referenced with other workers findings.

7.2 Materials and Methods

7.2.1 Species trials

The species trials described here are incorporated in the pair of *E.nitens* field trial plantings reported in Chapter 6. Details of trial locations, site preparation, experimental design, planting and assessment procedures are contained in Section 6.2.

Plantings contained 24 seedlots, comprising two provenances from each of 12 other *Eucalyptus* species, six from subgenus *Monocalyptus* and six from subgenus *Symphyomyrtus* , (see Appendix B) in addition to 101 open-pollinated *E.nitens* families (five families were combined so that there were only 97 seedlots). Most seedlots were from collections of five or more open-pollinated trees, and individual tree identification was retained at planting in 13 of the seedlots. Insufficient seedlings germinated from both provenances of *E.delegatensis, E. pauciflora* ssp. *pauciflora, E.fastigata* and four families of *E.nitens*. As there was insufficient time to stratify additional *E.delegatensis* and *E. pauciflora* ssp. *pauciflora* seed, two different *E.delegatensis* provenances and two provenances of *E.laevopinea* respectively, were chosen as replacements, from germinated seed available elsewhere. Additional *E.fastigata* and *E.nitens* seedlings were grown (in the same size paper pots) in the Botany Department glasshouse to suppliment those with insufficient seedlings. Unseasonally wet and cold weather during the first few weeks following transplanting resulted in atypically poor survival in approximately half of the seedlots, viz. 75% average survival c.f. usual survival of at least 95%.

Seedlings were visually assessed for frost damage in August 1985 and measured for height and diameter (10 cm above ground level) in April 1986. Diameters were measured in only six of the 24 replications at each site.

Estimates of relative frost resistance were obtained for 14 seedlots, comprising two *E.nitens* families and one provenance from each of the 12 other species, by artificially frosting discs from leaves sampled in August 1985 and February 1986. The two *E.nitens* families were R14 and S17 (see Appendix A), as in mid-winter these were found to be the most and least frost resistant families sampled from a three-year-old plantation (see Table 6.6). Leaves were collected from Hampshire on 31/07/1985 and from Racecourse approximately two weeks later for winter assessment, and from both sites on 26/02/1986 for summer assessment. Usually seven or eight trees from each seedlot were sampled on each

occasion. Hence, up to eight estimates of frost resistance were obtained for each seedlot at each site in winter, and usually four estimates for each seedlot at each site in summer. Due to extensive frost damage to *E.fraxinoides* and *E.laevopinea* at Racecourse, these species were sampled at Hampshire only.

Usually one leaf was collected from each tree where the required number of discs (usually four) could possibly be taken from that one leaf. Sampling as few leaves as possible was particularly critical at the time of winter assessment, because a number of species had relatively little foliage, e.g. *E.laevopinea* and *E.coccifera* . Multiple leaves were required for *E.coccifera, E.dalrympleana* ssp. *dalrympleana, E.johnstonii, Erubida* and *E.urnigera* , where usually two discs could be taken from each leaf, and *E.gunnii* (where each disc was taken from a separate leaf). Leaf storage and frosting methods were as described earlier (Section 6.2).

7.2.2 Grafted material

To check the relative frost resistance of *E.nitens*, *E.gunnii* and *E.perriniana* under controlled environmental conditions, and to assess the likelihood of any graft transmissable factor involved in frost resistance, reciprocal grafts were made between *E.nitens* and *E.gunnii,* and *E.nitens* and *E.perriniana.* As a control *E.nitens* was also grafted onto *E.nitens.* Spare seedlings from the field trials were used as root stocks and scions were obtained from two *E.nitens* clones and single *E.gunnii* and *E.perriniana* clones. A grafting technique similar to that of Cauvin (1981) was used. About 40% of all grafts with *E.nitens* scions succeeded, irrespective of the species of the stock, and most failure occurred where stocks had no foliage. Similarly, about 40% of grafts involving *E.gunnii* scions succeeded (though some failed after actively growing for three to eight months). All *E.perriniana* scions grafted onto *E.nitens* failed.

The frost resistance of foliage on both scions and stocks was assessed using leaf discs, approximately four months after grafts were made (all plants were growing in a glasshouse) and subsequently after four and eight weeks of hardening under controlled conditions (Table7.5). Plants were then dehardened for four months in a glasshouse, during which time the foliage of the stocks was removed leaving only scion foliage on all plants. The frost resistance of the scion foliage was then assessed using leaf discs after four weeks of hardening under controlled conditions (Table7.5).

7.2.3 Frost resistance of plantation trees of various ontogeny and size

To investigate the possible effects of plant ontogeny and size on levels of frost resistance, leaf discs were frosted from juvenile foliage collected from plantation trees of three ages. All trees were from the same open-pollinated *E.nitens* family (M06, Appendix A) and were planted on the same level site, within *c.* 400 m of each other. All leaves had recently fully expanded (presumably at the end of the previous summer) and were sampled from the same height above ground (*c.* 50 cm).

7.3 Results

7.3.1 Temperature variation

There were large differences in many temperature parameters between sites, between years and throughout each year (Figure 7.1). Overall, Hampshire was the milder site, particularly during late winter and early summer, e.g. mean maximum and minimum temperatures were *c.* 2°C warmer and frosts were less frequent and of shorter duration. On average for both sites, mean maximum and minimum temperatures ranged from *c.* 15 and 7°C respectively in summer to 6 and 1°C in winter. In addition, the frequency and duration of frost, and the daily duration of temperatures low enough for substantial hardening {≤ 6°C screen temperature, which corresponded to a temperature 10 cm above ground level of *c.* 4°C(see Appendix C)} reached minimum values in summer and maximum values in winter.

Frosts occurred at both sites within one month of planting. During their first year in the field, seedlings at Hampshire and Racecourse were subjected to *c.* 77 and 87 frosts respectively, with absolute minima (0.1 m above ground) of -7 and -10°C respectively. Visual assessments of frost damage were made on separate occasions at Hampshire and Racecourse (two weeks apart), *c.* seven and 20 days respectively after the absolute minima were recorded.

7.3.2 Initial survival

In May 1985 (4 months after planting) there was no significant difference (P>0.05) in the survival at Hampshire (80%) or Racecourse (82%), nor were there any significant differences amongst the species (Tables 7.1, 7.2). However, survival between species and sites, i.e. the interaction component, was extremely variable with the poorest survival of 58% for *E.regnans* at Hampshire and the best survival of 90% for *E.gunnii* at Racecourse.

7.3.3 Frost resistance

The mean frost damage scores for the species at each site are listed in Table 7.1 and the percentage seedlings within each frost damage class at Racecourse are depicted in Figure 7.2. On average seedlings sustained much more damage at Hampshire than at Racecourse (Table 7.2). Whilst the low level of damage at Hampshire was not sufficient to separate the species, there were large differences amongst the species at Racecourse (Table 7.1). For instance, *E.gunnii* was virtually undamaged at Racecourse whilst species such as *E.fastigata, E.regnans, E.laevopinea* and *E.fraxinoides* suffered more than 70% foliage damage on average. Within five of the species there were significant differences (P<0.05) between the two representative provenances at Racecourse (Table 7.1).

Assessment of mid-winter relative frost resistance using leaf discs also revealed

highly significant differences between the sites, species and their interaction (Table 7.2). On average, the trees at Racecourse were more frost resistant in winter, sustaining similar levels of leakage of cellular electrolytes at temperatures 1.3°C colder than for samples from Hampshire (Table 7.3). Levels of frost resistance differed by over 7°C between some species, e.g. *E.regnans* (-6.1°C) and *E.gunnii* (-13.3°C) at Racecourse. Some species such as *E.johnstonii, E.fastigata* and *E.regnans* had similar levels of frost resistance at both sites, whilst others such as *E.dalrympleana, E.gunnii, E.perriniana, E.rubida, E.coccifera* and *E.delegatensis* were on average 1.0°C hardier at Racecourse (Table 7.3, Figure 7.3).

In late-summer (13 months after planting) seedlings were on average 5°C less frost resistant than they had been the previous winter (Table 7.3). Even so, there were still highly significant differences amongst species (Table 7.2), with *E.perriniana, E.gunnii, E.dalrympleana* and *E.coccifera* exhibiting high levels of frost resistance. Both sites were characterised by similar levels of overall frost resistance in summer, although there was still a highly significant site-by-species interaction, i.e. some species were apparently more frost resistant at Racecourse (*E.coccifera*), others at Hampshire (*E.gunnii*) and still others equally resistant at both sites (*E.delegatensis*). There was a strong correlation between the mean level of frost damage assessed visually at Racecourse and the estimate of relative frost resistance using leaf discs (Figure 7.4a).

7.3.4 Survival after one year

There were highly significant differences between the sites and amongst the species in survival at 16 months after planting (Table 7.2). The average level of mortality in the 12 month period between the two assessments was three times as high at Racecourse as at Hampshire (Table 7.1). As the likelihood of plant death was found to increase with the level of damage visually assessed (Figure 7.5), the reduced survival at Racecourse is most likely largely attributable to the effects of greater frost damage. Between the two assessments, 207 plants died at Hampshire and 156 (75%) had been scored as having $\leq$10% leaf damage (damage score=1). The relatively large mortality in low damage classes may be associated with some other factor(s) since all subsequent frosts were much milder. Parts of the Hampshire site were subjected to periodic inundation from water and there was intense grass cover over the whole site by the end of the first years growth. There was very little weed competition and no waterlogging at Racecourse. Therefore, some of the mortality at Hampshire may be associated with waterlogging and grass competition.

7.3.5 Height and diameter growth

Height growth was significantly greater at Hampshire than at Racecourse, whilst mean diameters were similar at both sites (Table 7.4). There were highly significant differences in growth amongst and within the *E.nitens* provenances. The provenances from Victoria were on average 27 and 39 cm taller,and 0.6 and 0.8 cm greater diameter than the Southern N.S.W. and Northern N.S.W. provenances respectively. The Errinundra

provenance ("early-adult form") which is typically slower growing than the Western provenance with "juvenile-persistent form" (Pederick 1979) was in fact the tallest and largest diameter provenance at Hampshire. However, at Racecourse it was 10 cm shorter than the other Victorian provenances, yet still had equal greatest diameter (Table 7.4). Observations suggest that this was largely due to some loss of height, as a result of frost damage at Racecourse. Significant differences in mean height between families, within provenances, were evident in all six provenances at this early age (Table 7.4).

Mean heights and diameters amongst the species were also found to differ significantly (Table 7.2). All species were on average shorter and thinner than the *E.nitens*, although the best of these other species (viz., *E.dalrympleana* and *E.regnans*) were as large as the smallest *E.nitens* provenances, in terms of both height and diameter (Table 7.4). Those species from subgenus *Monocalyptus* were characteristically shorter and thinner than those from *Symphyomyrtus* (Table 7.4) but the differences were not significant (Table 7.2). Early height growth across *E.nitens* or within provenances of *E.nitens* was found to be poorly correlated with frost resistance (Figure 7.6). Height at Hampshire was used rather than height at Racecourse, since there was relatively little loss of height due to frost damage at Hampshire, unlike the situation at Racecourse.

7.3.6 Frost resistance of grafted material

The frost resistance of foliage from the grafted material was generally determined by the genotype of the foliage itself, irrespective of the genotype of the adjoining scion or stock (Table 7.5). This suggests that there was no obvious graft transmissible factor affecting frost resistance, since large differences in frost resistance of leaves were evident where species of widely differing frost resistance were grafted together. For instance, after eight weeks hardening, *E.gunnii* foliage was at least 0.6°C and up to 3.5°C more resistant than adjoining *E.nitens* foliage. The *E.nitens* scions hardened significantly more ($P<0.01$) in the absence of foliage on root stocks than previously with foliage on root stocks, viz., -6.1 and -4.4°C (i.e. mean of -4.0 and -4.7°C) respectively after four weeks hardening (Table 7.5).

7.3.7 Effects of plant ontogeny and size on frost resistance

The trees planted in 1981 were at least 1.0°C more frost resistant ($P<0.01$) than trees planted in 1984 or 1985 (Table 7.6). At all four frost temperatures there was a consistent ranking of planting dates, viz., in order increasing frost resistance (decreasing leakage of cellular electrolytes) 1985<1984<1981.

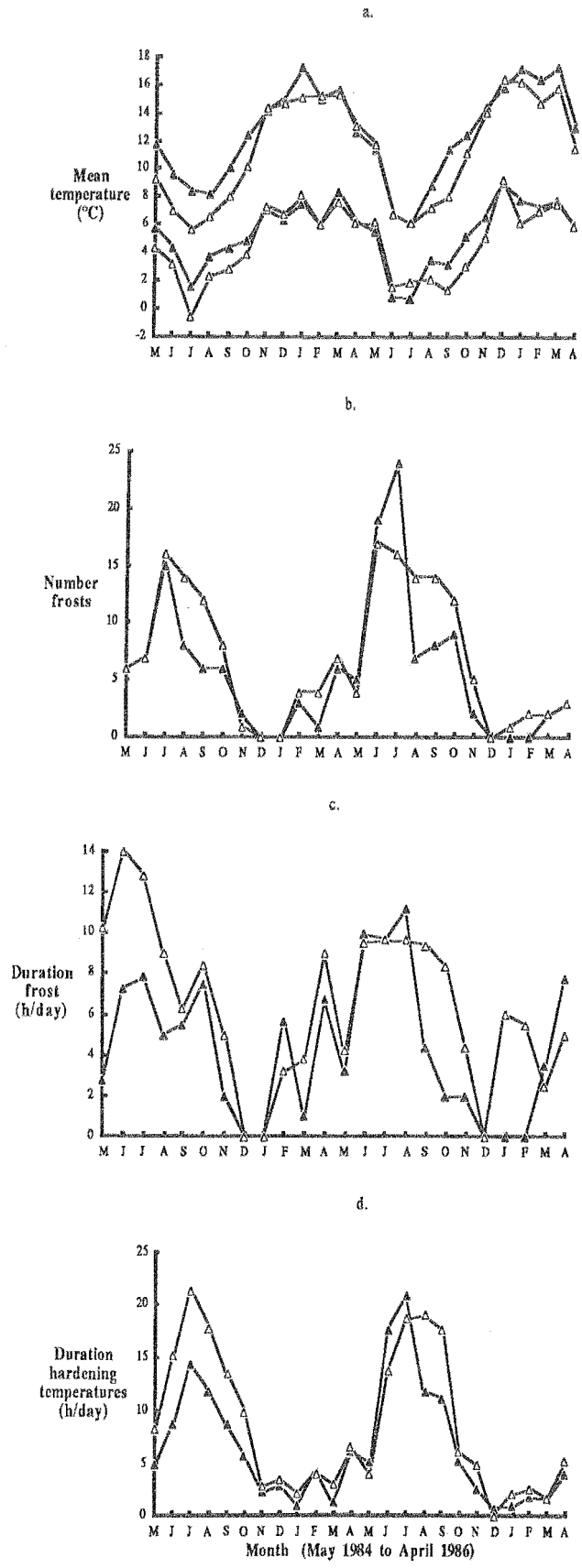


Figure 7.1 Mean monthly, (a) maximum and minimum temperatures, (b) number of frosts (only 28 days month^{-1}), (c) duration of frost (for those days on which a frost occurred) and (d) duration of temperatures ≤ 6°C, from thermohygrographic records at Hampshire (▲) and Racecourse (△).

Table 7.1 Mean survival (4 and 16 months after planting) and frost damage (eight months after planting) for 13 species at Hampshire (Ham.) and Racecourse (Rac.). Significant differences in frost damage between provenances within a species are indicated for P<0.05 (*), P<0.01(**) and P<0.001(***).

Species	Survival (4 mth)		Frost damage		Survival(16mth)	
	Ham. (%)	Rac. (%)	Ham. (0-5)	Rac. (0-5)	Ham. (%)	Rac. (%)
	E.nitens					
Rubicon	80	86	0.4	1.4	78	81
Toorongo	84	86	0.4	1.7	80	73
Macalister	86	87	0.4	1.5	82	78
Errinundra	76	82	0.5	2.5	67	51
Southern N.S.W.	83	84	0.4	2.5	74	44
Northern N.S.W.	81	82	0.3	1.4	67	56
E.nitens mean	82	84	0.4	1.4***	75	63
	Subgenus *Symphyomyrtus*					
E.dalrympleana	88	71	0.4	1.3*	83	58
E.gunnii	71	90	0.0	0.1	69	83
E.johnstonii	75	73	0.3	2.3	71	50
E.perriniana	81	62	0.2	0.4	77	58
E.rubida	69	71	0.5	1.4*	58	54
E.urnigera	79	85	0.1	1.7**	73	71
Subgenus mean	77	75	0.3	1.2	72	62
	Subgenus *Monocalyptus*					
E.coccifera	69	81	0.1	0.7***	62	79
E.delegatensis	62	71	0.2	1.4	56	62
E.fastigata	79	75	0.7	3.1***	64	40
E.fraxinoides	73	60	1.3	4.6	52	0
E.laevopinea	88	62	1.2	4.0	62	12
E.regnans	58	75	0.5	3.5	54	25
Subgenus mean	72	71	0.8	2.9	59	36
Mean	80	82	0.4	2.1	59	36

Table 7.2 Levels of significance from ANOVA for survival, frost damage, frost resistance (T50), height and diameter; not assessed (NA), P>0.05 (NS), P<0.05 (*), P<0.01(**) and P<0.001(***).

Level of comparison	Survival 4 mth	16mth	Frost† damage	Height	Diameter	T50†† Winter	T50†† Summer
		E.nitens only					
Site	*	***	***	***	NS	NA	NA
Provenance	**	***	***	***	***	NA	NA
Interaction	NS	***	NS	**	**	NA	NA
		All species					
Site	NS	***	***	***	NS	***	NS
Species	NS	***	***	***	***	***	***
Site*Species	NS	**	***	***	NS	***	***
Subgenus	NS	***	NS	NS	NS	NS	NS
Site*Subgenus	NS	NS	*	NS	NS	NS	NS

†, based on arc-sine square-root transformation of percentage damage

††, temperature resulting in 50% leakage of cellular electrolytes

Table 7.3 Mean frost resistance of 13 species at Hampshire and Racecourse determined using leaf discs. Given are frost temperatures resulting in 50% leakage of cellular electrolytes (°C).

Species	July/August 1985[A]		February 1986[B]	
	Hampshire	Racecourse	Hampshire	Racecourse
	E.nitens			
Rubicon (R14)*	-8.4	-8.8	-3.5	-3.1
Southern N.S.W. (S17)*	-7.4	-8.2	-3.2	-2.6
	Symphyomyrtus			
E.dalrympleana	-7.9	-9.8	-4.0	-4.6
E.gunnii	-11.4	-13.3[C]	-5.3	-4.0
E.johnstonii	-8.1	-8.0	-3.5	-2.9
E.perriniana	-9.1	-12.3	-5.0	-4.4
E.rubida	-7.0	-10.1	-4.5	-4.0
E.urnigera	-8.4	-9.1	-3.6	-3.6
Subgenus mean	-8.6	-10.4	-4.3	-3.9
	Monocalyptus			
E.coccifera	-10.1	-11.4	-3.3	-4.8
E.delegatensis	-8.4	-9.6	-3.5	-3.6
E.fastigata	-7.8	-7.1	-3.6	-3.0
E.fraxinoides	-6.4	NA[D]	-2.6	NA
E.laevopinea	-7.1	NA	NA	NA
E.regnans	-5.9	-6.1	-3.6	-2.9
Subgenus mean	-7.6	-8.6	-3.5	-3.6
Mean	-8.3	-9.6	-3.9	-3.7

A, samples from 7 or 8 trees seedlot^{-1} collected 31/07/1985 (Hampshire) and 18/08/1985 (Racecourse)

B, samples from 3 or 4 trees seedlot^{-1} at each site collected 27/02/1986

C, an underestimate as discs from all trees had <50% leakage of cellular electrolytes at the minimum temperature of -13.3°C

D, not available because of insufficient healthy trees

*, family identification (see Appendix A)

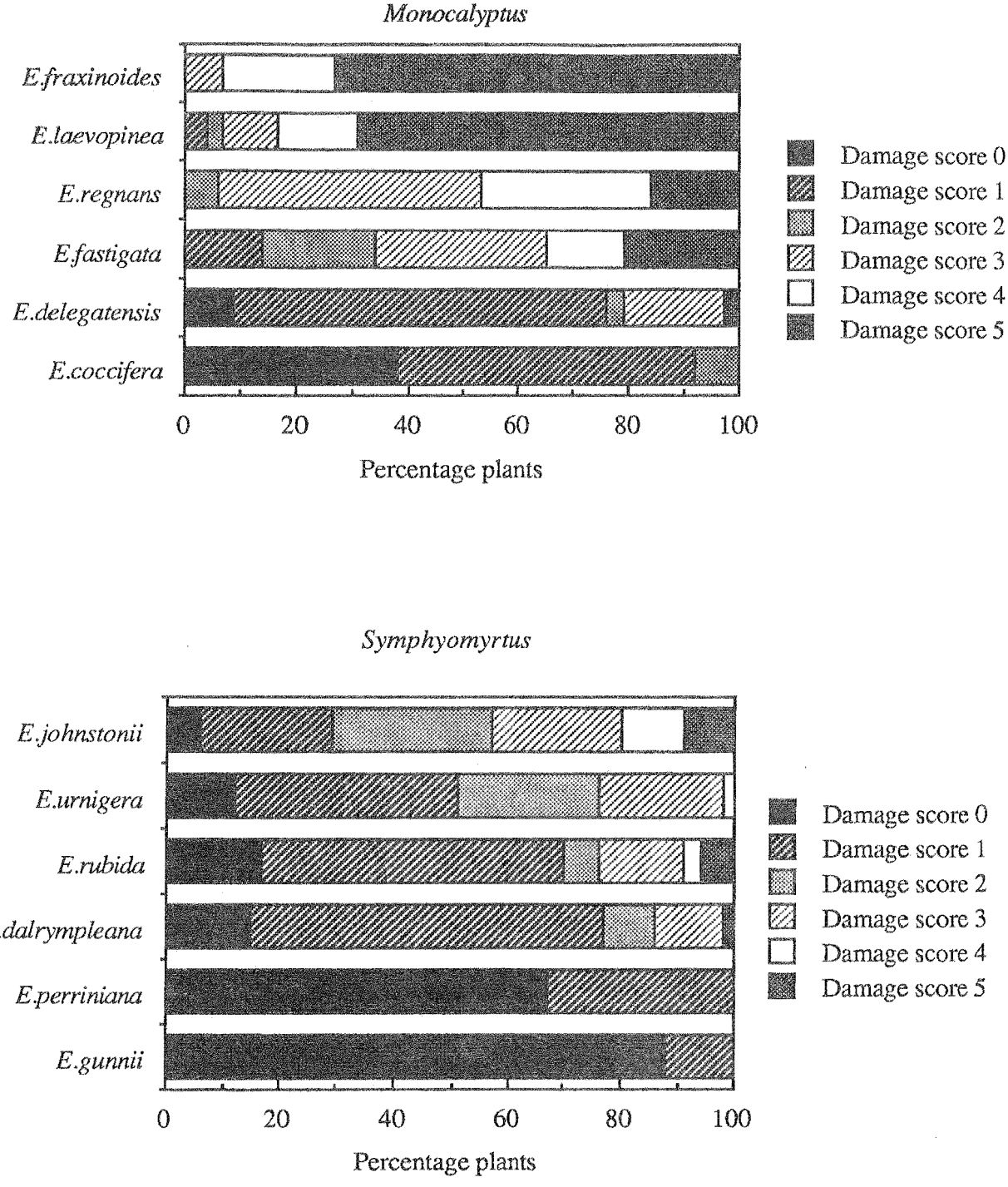


Figure 7.2 Percentage of eucalypt seedlings in each of six frost damage classes, for six species from *Monocalyptus* and six species from *Symphyomyrtus*. The data for each species were collected from two provenances.

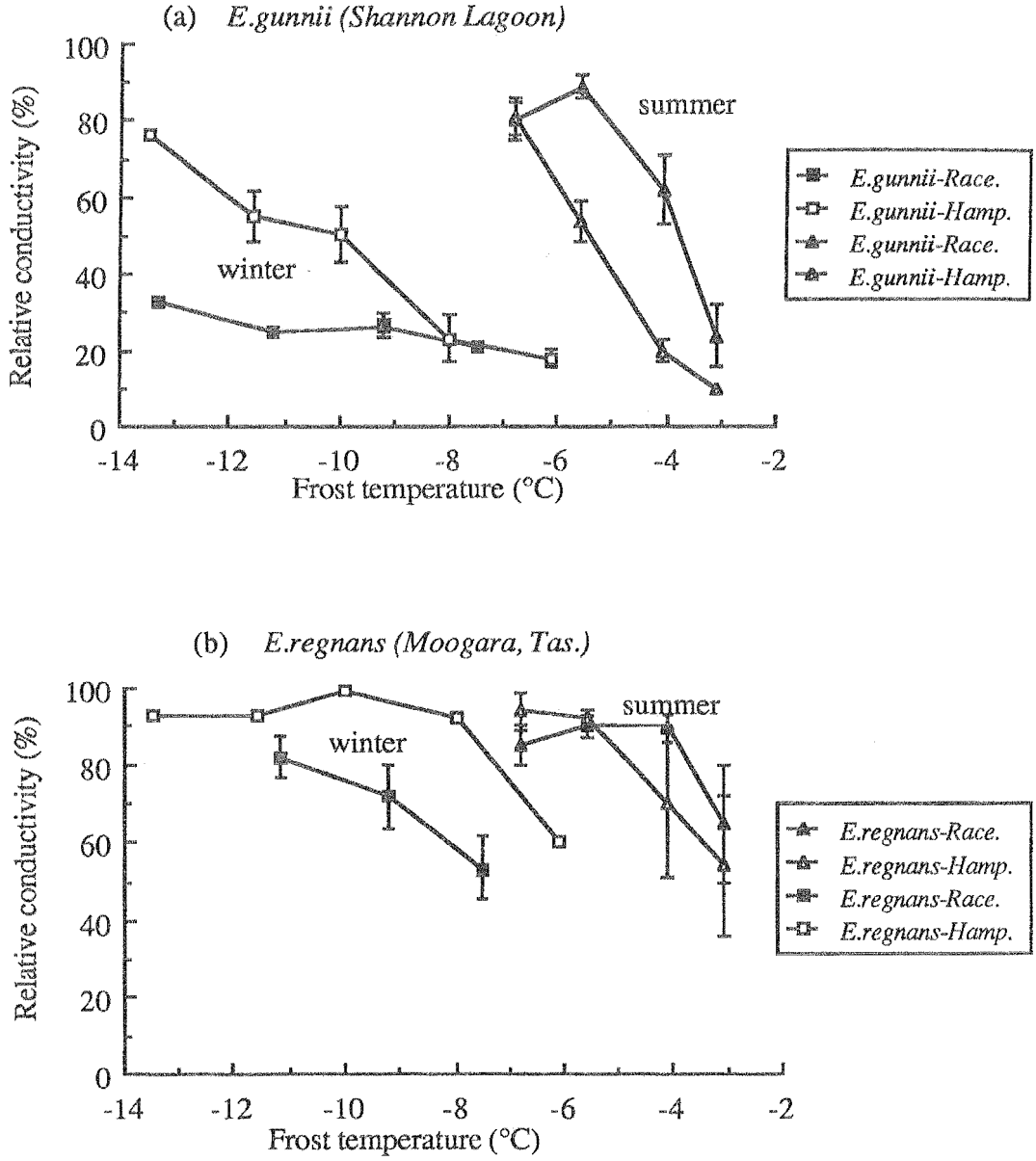


Figure 7.3 Relationships between relative leakage of cellular electrolytes and frost temperature, for leaf discs from (a) *E.gunnii* and (b) *E.regnans*. Vertical bars are± S.E. (≥ 2%).

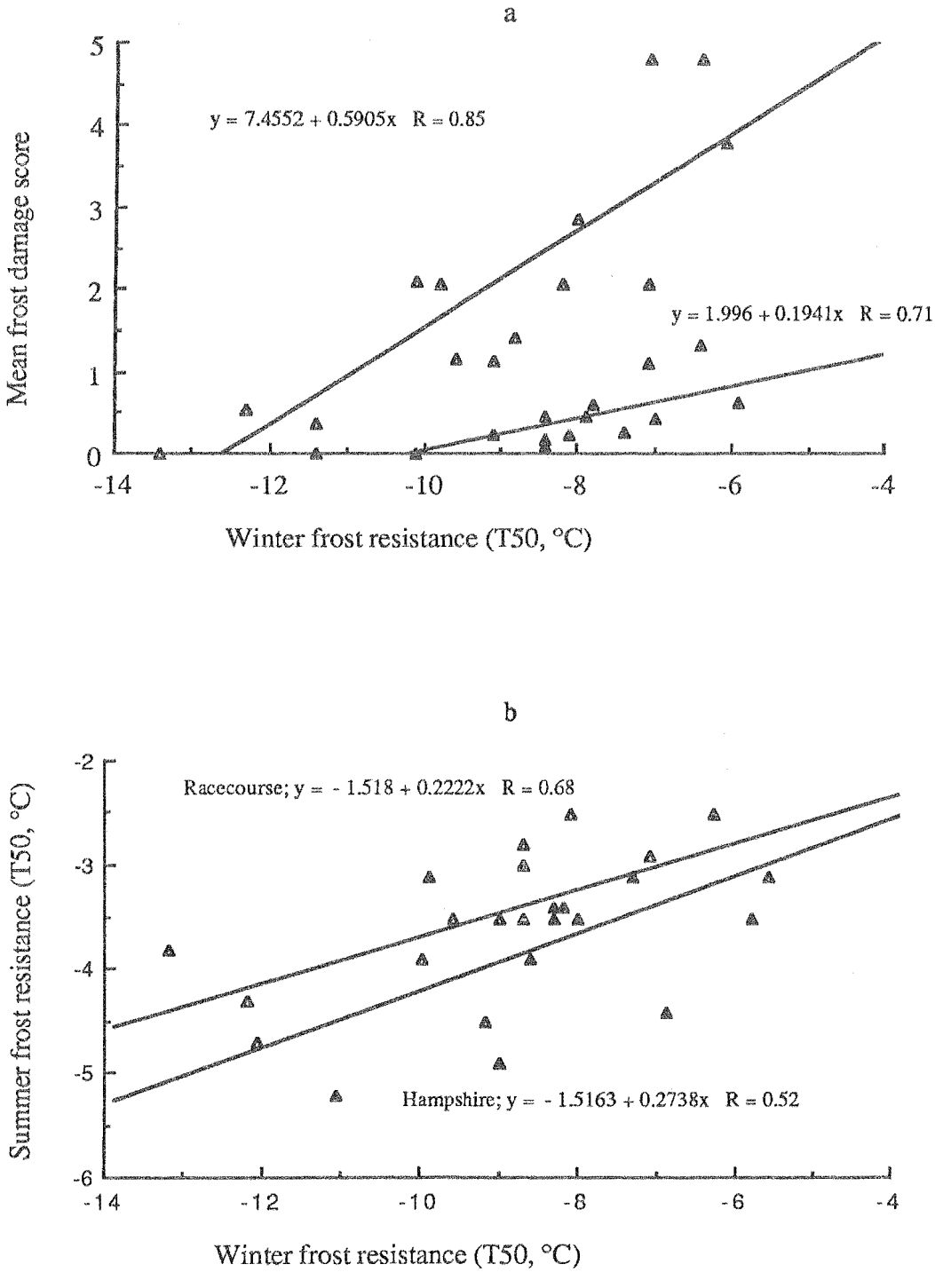


Figure 7.4 Relationships between winter frost resistance and (a) mean frost damage (assessed visually), and (b) summer frost resistance, for eucalypt seedlings at Hampshire (▲) and Racecourse (△).

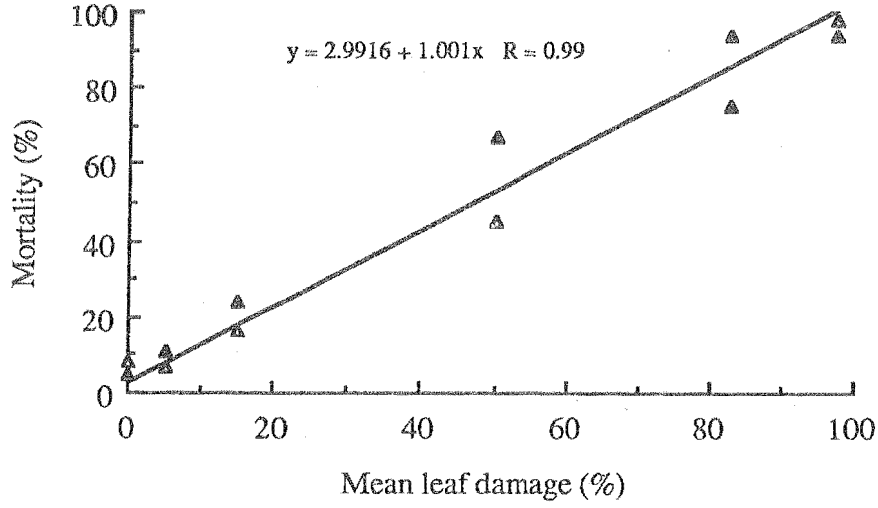


Figure 7.5 Relationships between percentage mortality and mean percentage leaf damage for all eucalypt species at, Hampshire (▲) and Racecourse (△).
Mean leaf damage is the midpoint of the range of damage,in each leaf damage class.

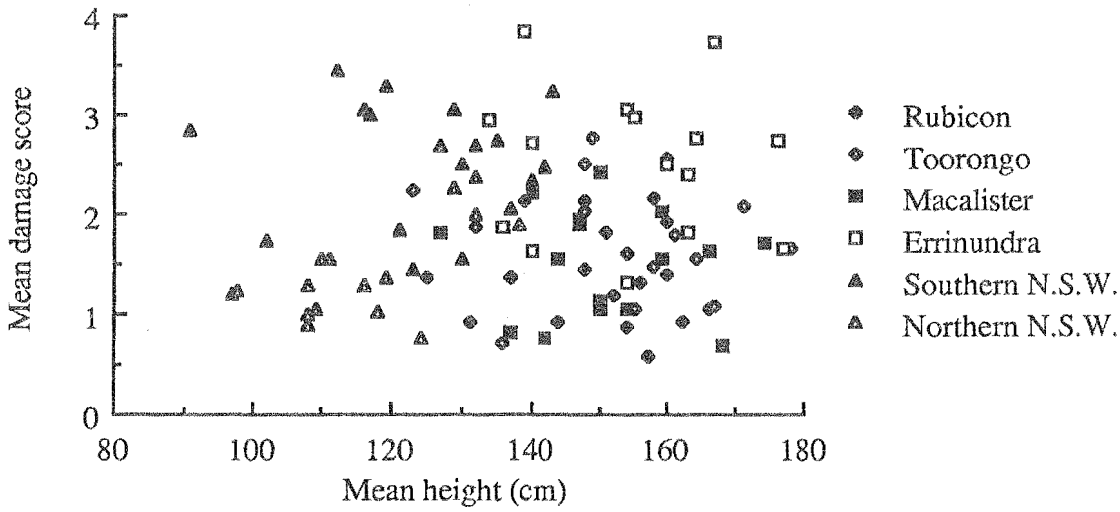


Figure 7.6 Relationships between mean frost damage (at Racecourse) and mean height (at Hampshire) for all 97 *E.nitens* families.

Table 7.4 Mean height and diameter according to species at Hampshire and Racecourse. Significant differences for height amongst families within a *E.nitens* provenance and between provenances within a species are indicated for P<0.05 (*), P<0.01(**) and P<0.001(***).

Species	Height (cm)		Diameter (cm)	
	Hampshire	Racecourse	Hampshire	Racecourse
	E.nitens			
Rubicon ***	149	92	2.1	2.4
Toorongo ***	154	92	2.1	2.3
Macalister ***	151	93	2.4	2.4
Errinundra ***	155	80	2.4	2.4
Southern N.S.W. ***	127	67	1.6	1.6
Northern N.S.W. *	115	80	1.4	1.5
E.nitens mean	142	82	2.0	2.1
	Subgenus *Symphyomyrtus*			
E.dalrympleana	129	72	1.5	1.0
E.gunnii	126	77	0.9	1.1
E.johnstonii	118	57	1.2	1.2
E.perriniana	103	74	0.8	1.3
E.rubida	117	59	1.1	1.0
E.urnigera *	151	60	1.4	1.2
Subgenus mean	124	66	1.2	1.1
	Subgenus *Monocalyptus*			
E.coccifera	75	44	0.7	0.8
E.delegatensis **	91	48	1.0	1.0
E.fastigata	95	41	1.0	0.8
E.fraxinoides	69	-	0.6	-
E.laevopinea	60	24	0.6	0.4
E.regnans	122	49	1.6	0.9
Subgenus mean	85	41	0.9	0.6
Mean	136	78	1.9	1.9

Table 7.5 Relative frost resistance (T50, °C) of grafted *E.nitens*, *E.gunnii* and *E.perriniana*. The same plants were hardened under either 18°C day(8 h)/2°C night (*) or 25°C(glasshouse 8 h) day/3°C night (**). Values with similar letters at a given duration of hardening are not significantly different (P<0.01).

Foliage	Weeks of hardening			
	0*	4*	8*	4**
E.nitens root stocks	-2.8b	-4.4c	-6.6c	-
E.gunnii root stocks	-3.2a	-5.3ab	-7.6b	-
E.perriniana root stock	-3.0ab	-6.5a	-9.1a	-
E.nitens scion on *E.nitens*	-3.0ab	-4.0c	-7.0bc	-6.2a
E.nitens scion on *E.gunnii*	-2.7b	-4.7b	-7.1bc	-6.0a
E.nitens scion on *E.perriniana*	-2.9b	-4.8b	-6.0c	-
E.gunnii scion on *E.nitens*	-3.4a	-6.4a	-10.1a	-
Mean ± S.D.	-2.9 ± 0.43	-4.8 ± 1.02	-7.3 ± 1.29	-6.1 ± 0.70

Table 7.6 Relative frost resistance of trees of three ages from the same single open-pollinated family of *E.nitens*, growing on the one site in north-western Tasmania (near the Hampshire trial, Figure 3.1). Given are (mean ± S.E.) relative conductivities of the bathing medium containing frosted leaf discs, and frost temperatures causing 50% leakage of cellular electrolytes (T50). Frost resistance was evaluated in September 1986.

Year of planting	Relative conductivity (%)				T50 (°C)
	-6.4°C	-7.5°C	-9.0°C	-10.5°C	
1981	39.3 ± 2.2	50.7 ± 3.6	62.1 ± 3.8	64.8 ± 2.0	-8.0 ± 0.3
1984	48.6 ± 4.0	56.4 ± 4.1	76.9 ± 3.0	69.2 ± 1.8	-7.0 ± 0.2
1985	55.7 ± 5.0	68.5 ± 4.6	84.8 ± 3.4	74.8 ± 1.8	-6.7 ± 0.2

7.4 Discussion

Large differences were found in the relative frost resistance amongst the species tested, particularly in mid-winter (Table 7.3). Although frost damage was severe enough at Racecourse to discriminate between species, the levels of damage did not indicate the absolute differences in relative frost resistance, e.g. how different were *E.gunnii* which was largely undamaged and *E.fraxinoides* which was largely killed? However, artificial frosting of leaf tissue enabled these differences to be elucidated and showed that in winter some species differed by over 7°C in level of frost resistance. The validity of the leaf disc technique is supported by the significant correlation ($P<0.05$) between the mean level of frost damage and the estimates of relative frost resistance using leaf discs (Figure 7.4a). This contributes to the growing body of data affirming the general applicability of the leaf disc technique for assessing frost resistance in eucalypts, e.g. Harwood (1981); Hallam (1986); Raymond *et al.* (1986); and this study (Figure 4.7, Section 5.3). Such findings support the view that the leaf disc method offers a suitable and superior alternative to the otherwise uncertain, more expensive, destructive and slower option of establishing field trials in frost-prone areas, for screening and selection of species, provenances and/or families for frost resistance.

However, the sensitivity of the leaf disc method for separating species is markedly influenced by the season of sampling (Table 7.3). Whilst it may be possible to identify relatively frost resistant and sensitive species in summer, when plants are generally at their least level of hardiness, this may not be always so (Hallam 1986). The greatest differences amongst species are likely to be found when plants are at a higher overall level of hardiness. The season at which plants are tested may largely depend on whether selection is on the basis of resistance to out of season (spring, summer or autumn) or winter frosts. Analysis of seasonal variation in frost resistance of *E.nitens* families (Table 6.6) revealed significant genotype by season interaction. Part of this was possibly due to some families dehardening sooner than others. Should commencement of growth after winter and dehardening be linked, such season by level of hardiness interactions may be even greater between species, particularly when species which have different temperature optima for growth (see Paton 1980) are compared.

The relative frost resistance of specific seedlots in this study favourably agrees with that from some overseas research. For instance, the *E.dalrympleana* provenance from the Cotter Hut area of the A.C.T. (Australian Capital Territory, see Appendix B) and the *E.nitens* families from 1400 m altitude in Anembo State Forest (Southern N.S.W.), had mean damage scores at Racecourse of 0.7 and 1.8 respectively. In a trial with the same seedlots in Natal, South Africa, *E.dalrympleana* received only 47% of the damage that *E.nitens* did (Nixon 1983). In addition, the superior frost resistance of the Oberon (N.S.W.) provenance compared with the Bombala (N.S.W.) provenance of *E.fastigata* used here, has also been demonstrated in New Zealand (Wilcox 1982c).

The relative ranking of the species (according to their frost resistance) is generally in

good agreement with that reported elsewhere, i.e. *E.gunnii* is very frost resistant, *E.nitens* is moderately frost resistant and *E.regnans* is less frost resistant (Evans 1983). Clearly there is wide scope to select for this trait in the genus *Eucalyptus*. Data indicating the relative ranking of eucalypt species is vitally important to those wishing to establish plantings in frost prone sites. The findings from these studies, and particularly the ability to determine relative differences amongst species using leaf discs, highlights the extent of differences between some species (see Table 7.7).

Early results show significant provenance variation in height and diameter growth and also intraprovenance variation in height growth of *E.nitens* (Tables 7.2 and 7.3, Figure 7.6). Highly significant differences between *E.nitens* provenances have been reported both on the Australian mainland and overseas, though not at such an early age, e.g. two years after planting in Victoria(Pederick 1979),three years in New Zealand (New Zealand Forest Service 1980) and South Africa (Darrow 1984), and, four years in South Australia (Cotterill *et al.* 1985). However, the comparative growth rate of the provenances at the early stages in this study may not be typical of long-term growth rates. For instance, the Errinundra provenance was the tallest provenance at Hampshire,but by two to four years after planting it is generally the least vigorous provenance in south-eastern Australia and New Zealand (Pederick 1979; New Zealand Forest Service 1980; Cotterill *et al.* 1985; Tibbits 1986). Pederick (1979) found that seedlings of the Errinundra provenance were tallest at planting. The tall height of the Errinundra provenance (at Hampshire) is possibly due to its relatively longer internodes. This has been previously demonstrated in potted seedlings (Table 6.2, and section 6.2.3). In the field trials, trees of the Errinundra provenance were generally characterized by longer internodes than the other provenances (personal observations). Although trees of the Errinundra provenance were on average slightly taller than those of the Western provenance (Rubicon, Toorongo and Macalister) they were probably charactersied by much less leaf area because of fewer nodes and smaller leaves (see Pederick 1979). These differences and the early transition to adult foliage in the Errinundra provenance could partly explain the slower growth of the "early-adult form" in subsequent years (see Pederick 1979).

Early results also show significant species variation in height and diameter growth (Table 7.4). *E.nitens* was the tallest species overall, although *E.urnigera, E.dalrympeana* and *E.gunnii* had heights that were at most only 20 cm less than *E.nitens* at Hampshire (the site where frost damage had little effect on height). However, *E.nitens* was clearly the most productive species because of its greater diameter. The mean height and diameter of the smallest species, *E.laevopinea,* were only 42 and 30% of that of *E.nitens*. The superior early growth of *E.nitens* compared with species such as *E.regnans, E.fraxinoides, E.delegatensis, E.fastigata* and *E.laevopinea* was also demonstrated in South Australia on a much more productive site, where mean height at 18 months was 3.7 m. Generally, those species from subgenus *Monocalyptus* tested here were less vigorous than those from subgenus *Symphyomyrtus*. Indeed, Turnbull and Pryor (1978) conclude that the *Monocalyptus* "group is more difficult to establish in plantations and is often more sensitive to environmental conditions....than other subgeneric groups."

Although the longer-term growth potential of the various species is not clear, it would seem that *E.nitens* should remain one of the most productive species . The occurrence of unusually severe frosts (e.g. -15°C), such as may happen at Racecourse, may mean that only more frost resistant species may be more productive, e.g. *E.gunnii*. Estimates of relative productivity indicate that some species may be equally as productive as some of the poorer provenances of *E.nitens* (Table 7.8). Clearly, the ultimate selection of species and provenances will depend on the relative importance given frost resistance and growth and any other traits sought after.

The data show that frost resistance of open-pollinated *E.nitens* families is uncorrelated with early height growth (Figure 7.6). Similar findings have been reported for *E.regnans* based on open-pollinated families (Wilcox *et al.* 1980) and provenances (Griffin *et al.* 1982b). This indicates that selection for improvement of both traits is possible in *E.nitens* and maybe also in a number of other eucalypt species. The large difference in frost resistance between some of the more frost resistant species and *E.nitens*, may mean that controlled breeding using these species may be one tool of producing genotypes, that are characterised by reasonably high levels of frost resistance and growth. Such a programme is already underway in France (Potts and Potts 1986).

There are various data indicating that levels of frost resistance and damage may be influenced by the relative development of the plant (size and ontogeny). Meskimen (1983) concluded that faster growing seedlots of *E.grandis* received less damage than slower growing seedlots because their foliage was largely above the coldest air layer. In this study, *E.laevopinea* received greater leaf damage at Racecourse than *E.regnans* although leaf disc estimates of frost resistance at Hampshire suggest that *E.laevopinea* was 1°C more frost resistant than *E.regnans*. Vertical stratification of air temperatures was recorded at both sites with temperatures at ground level being about 1°C colder than those at 40 cm above ground. This effectively means that taller seedlings (e.g. *E.regnans*) were on average subjected to milder temperatures than smaller seedlings, although lower sections of all seedlings would have been exposed to similar temperatures, other things being equal. In addition, *E.nitens* seedlings from the Northern N.S.W. provenance,were characterised by significantly lower survival after the first winter than the other provenances that were considered equally frost resistant, e.g. Rubicon, Toorongo and Macalister. Seedlings from Northern N.S.W. were on average smaller than those from the other provenances (visual observations at assessment, Table 7.4). This warrants further study, since significant height by damage interactions may preclude the leaf disc method alone to determine frost resistance in genotypes which differ greatly in growth.

The greater frost resistance of *E.nitens* scions in the absence of foliage on root stocks (Table 7.5) may be due to a relative increase in the vigour of the scion and/or the different growth treatments used. The scions hardened after foliage from root stocks had been removed were grown under warmer daytime conditions with much higher light intensities, than when they were previously hardened (Table 7.5). However, the 1.7°C difference in frost resistance after four weeks hardening is unlikely to be due to different growth treatments alone, since the difference in mean T50 values between two similar

treatments with potted seedlings was only 0.7°C (Figure 5.3). Additional evidence that the difference may be due to changes in vigour of the scion is the large variation in frost resistance of *E.nitens* scions in the initial hardening experiment. In particular, after eight weeks hardening, one of the scions had a T50 of -8.5°C whilst the other scions (all grafted from the same clone, with three leaves assessed from each scion) had T50 values of -6.0, -6.6, -6.8 and -7.2°C. This more resistant scion was on a *E.gunnii* stock which only had a small shoot (10 cm), whilst the other scions were on stocks (all three species) with shoots at least 50 cm. It is interesting to note that the overall rate of hardening (during four weeks) of the *E.nitens* in the first and second hardening treatments were 0.4 and 0.8°C per week respectively. These rates are in good agreement with that found in previous experiments (see Chapter 5).

There are also indications that plant ontogeny may have an important influence on relative frost resistance (Table 7.6). In addition, the two *E.nitens* families sampled at these trials were less frost resistant than two year older trees similarly assessed in the progeny trial in winter (Table 7.3 c.f. Table 6.6). Although larger diameter leaf discs were used when sampling the field trials, the effect should have been to increase the relative frost resistance of samples from the species trials (see Figure 4.6). These differences may not be due to ontogeny *per se* but may be related to its effects on plant vigour,e.g. root and shoot biomass. However, there are a number of other differences amongst the trees which could account for some of the variation in frost resistance. Leaves were sampled from the same height but the temperature microclimates of the three age groups may have been quite different. In particular, the 1981 planting had a closed canopy, whilst the canopies of the 1984 and 1985 plantings were quite open. This may mean that the younger trees may be subjected to lower minima. This should tend to increase their level of frost resistance (see Paton 1980). However, the insulating effect of the canopy of the1981 planting may also mean that the lower foliage was more shaded and did not warm up as quickly during the day, as leaves on younger trees. This may mean that leaves were exposed to hardening temperatures for a greater part of the day. Such a situation should increase frost resistance, since it was found that an increase in the daily amount of hardening temperatures resulted in an increase in the frost resistance of potted seedlings (Table 5.2). The relative effects of plant age, shading, minimum temperature and duration of hardening temperatures need to be better understood and appropriate experiments could be designed to assess these effects. This is particularly important where assessments of the relative frost resistance of planted trees involves sampling trees of various ages and stand structures.

Table 7.7 Relative winter frost resistance of *Eucalyptus* species. (Modified from Evans 1983), with additional sources being this study, Martin (1948), Nixon (1983), Menzies *et al.* (1981), Davidson and Reid (1985), Hallam (1986).

Subgenus *Monocalyptus*	Subgenus *Symphyomrytus*
-15 to -18 °C *	
E.pauciflora ssp. *debeuzillei*	*E.gunnii* ssp.*gunnii E.parvifolia*
E.pauciflora ssp. *niphophila*	*E.gunnii* ssp.*archeri*
-12 to -15°C	
E.coccifera	*E.perriniana E.glaucescens*
	E.vernicosa E.pulverulenta
-9 to -12°C *	
E.delegatensis	*E.bridgesiana E.nitens E.johnstonii E.urnigera*
	E.dalrympleana E.macarthuri E.melliodora
	E.aggregata
-6 to -9°C *	
E.fastigata E.laevopinea E.amygdalina	*E.viminalis E.grandis E.ovata E.camaldulensis*
E.pulchella E.regnans E.obliqua	*E.saligna E.cordata E.globulus E.leucoxylon*
E.fraxinoides	*E.lehmanii*
-3 to -6°C *	
E.preissiana E.sepulchralis	*E.sideroxylon E.grossaE.caesia E.forrestiana*
	E.torquata

*, Frost temperature range over which damage usually passes from slight to severe

Table 7.8 Estimated relative productivity* (at 16 months after planting) of *E.nitens* provenances and 12 other *Eucalyptus* species at the two field trials.

Species	Relative productivity (%)	
	At Hampshire	At Racecourse Plains
E.nitens provenances		
Rubicon	71	58
Toorongo	74	51
Macalister	100†	59
Errinundra	98	22
Southern N.S.W.	35	11
Northern N.S.W.	23	14
Subgenus *Monocalyptus*		
E.coccifera	4	2
E.delegatensis	8	7
E.fastigata	5	4
E.fraxinoides	1	0
E.laevopinea	11	1
E.regnans	12	9
Subgenus *Symphyomyrtus*		
E.dalrympleana	23	8
E.gunnii	12	9
E.johnstonnii	12	8
E.perriniana	5	14
E.rubida	11	5
E.urnigera	26	10

*approximately estimated as (mean tree volume x trees ha^{-1}) ÷ maximum († above)

CHAPTER 8

CONTROLLED BREEDING EXPERIMENTS

8.1 Introduction

Eucalyptus species are planted in many countries of the world (Brown and Hillis 1978). In both hemispheres, the preference for this genus at latitudes of less than 40°, is largely attributable to fast growth rates over a range of sites (Eldridge 1978). In addition, many fast growing species of *Eucalyptus* are characterized by desirable wood properties making them suitable for fuel wood (Eldridge 1983) or pulp and paper manufacture (Higgins 1978), respectively. In many of these overseas countries, programmes of genetic improvement to increase the yield and other desirable traits are well underway , e.g. Brazil (Campinhos 1980).

Until recently , only a small part of the tree breeding efforts within this genus had been undertaken in Australia. This is undoubtedly due to the very small contribution of plantation-grown eucalypts to wood production in Australia, relative to that from *Pinus radiata* D.Don., where breeding programmes are well developed. Until recently, most of the Australian *Eucalyptus* breeding efforts have been at the initial stages of genetic improvement programmes, i.e. identifying better species and provenances (see Eldridge 1975). Some examples are, *E.nitens* (Pederick 1979,1985), *E.regnans* (Griffin *et al.* 1982b), *E.obliqua* (Brown *et al.* 1976; Matheson *et al* 1986) and *E.delegatensis* (Boland and Dunn 1985). However, in Tasmania, the state with the largest *Eucalyptus* plantation programme, breeding programmes are progressing towards the production of genetically improved seed, by selecting better individuals within the better provenances of *E.globulus* ssp. *globulus* and *E.nitens* and the establishment of seed production areas (Tibbits 1986). The production of genetically improved eucalypt seed is also undertaken in Victoria for *E.regnans* (Cameron and Kube 1980a,b).

Genetically improved material is often produced from orchards of various designs (see Eldridge 1975), where seed may be obtained by controlled-pollination. However, of fundamental importance to efficient controlled-pollination, is a sound knowledge of the breeding systems of the species (Eldridge 1978). The definition of breeding system used by Lewis and John (1972) (cited by Eldridge 1978) is adopted, viz., 'all variables (except mutation) which affect genetic relationships of gametes that fuse' during sexual reproduction. A sound knowledge of the reproductive biology, and self and outcrossing compatibility, of species is particularly important in controlled-pollination programmes. The success of

breeding efforts with *E.globulus* spp. *globulus* and *E.nitens* will depend to a considerable extent on how well these aspects are understood in each species. Post-anthesis floral development has been comprehensively studied in some eucalypts, e.g. *E.grandis* (Hodgson 1976a,b,c) and *E.regnans* (Griffin and Hand 1979). However, *E.grandis* and *E.regnans* differed markedly with respect to parthenocarpy and the optimum time for pollination. The breeding systems of *E.globulus* ssp. *globulus* and *E.nitens* may be somewhat different from that of *E.grandis*, which naturally occurs in a very different climate, and *E.regnans*, which belongs to a different subgenus {following the informal classification of Pryor and Johnson (1971)}.

Controlled pollination studies were conducted during 1984, 1985 and 1986 on both *E.globulus* ssp. *globulus* and *E.nitens*. The purpose of these studies was twofold, viz.:-

(1) to examine various aspects of floral development following anthesis,which were likely to affect capsule and seed set, particularly the timing of stigma receptivity in *E.globulus* ssp. *globulus*, and

(2) to attempt a number of intraspecific and interspecific crosses in both species, with the view to looking at self and outcrossing compatibility and the heritability of a range of characters,with particular reference initially to morphological characters and frost resistance.

8.2 Methods of controlled pollination

The two species are vastly different in floral morphology (see Figures 8.1 and 8.2). Inflorescences are axillary and simple in each species, but with single glaucous buds (2.5 x 2.0 cm) in *E.globulus* ssp. *globulus* (Figure 8.2) and green umbels, usually with seven flowers (each flower *c.* 0.7 x 0.3 cm), in *E.nitens* (Figure 8.1). In Tasmania, *E.globulus* ssp. *globulus* usually flowers from late winter to early summer, whilst *E.nitens* almost always flowers only during mid to late summer. In both species bud primordia appear first visible to the naked eye about 12 months before flowering, and seed is ripe approximately 12 months following flowering (Figure 8.3).

8.2.1. Emasculation

At the time of anthesis, the inner operculum lifts from the hypanthium at the calycine ring. The time at which the operculum had started to lift from the hypanthium was designated as "day 0", and will hereafter be termed anthesis. Although this is technically incorrect, as anthesis is characterised by dehiscence of the anthers, it was a clearly identifiable stage of floral development and was very close to the actual time of anthesis. Flowers at this stage of development were selected, and their opercula and stamens carefully removed (emasculated) by cutting the tissue at the staminal ring. The branches containing these flower buds were then carefully placed in 45 cm X 23 cm bags, manufactured from non-woven polyester (Duraweld P.B.S.), and then effectively isolated by sealing the necks of the bags with non-absorbent cotton wool and a plastic-coated wire tie. Unopen flower

buds were also bagged separately to assess the ease of self pollination. An average of five and 20 flower buds were enclosed in each bag, for *E.globulus* ssp. *globulus* and *E.nitens* respectively.

In *E.globulus* ssp. *globulus*, there is no visible change in the colour of the operculum at anthesis. Typically, the inner operculum is completely shed and stamens expand, one day after the inner operculum begins to lift. All flower buds on each selected branch were generally not at the same stage of development. Flowers with opercula visibly lifting were emasculated by cutting (with a scalpel) through the base of the anthers at the staminal ring. Previously opened flowers were removed and unopen flowers were left untreated. Unemasculated, unopen buds were inspected every second day and emasculated when their opercula began to lift, before stamens could expand and anthers shed pollen. A clear plastic window (15 x 8 cm) along the length of each bag enabled easy inspection. In a few cases, flower buds which had not started to shed their operculum were emasculated to see whether their stigmas became receptive at the same time as those emasculated at anthesis. Measurements and observations were sometimes made on the appearance of the stigma, style and other aspects of emasculated buds and a number of open-pollinated flowers,at two-day intervals.

In *E.nitens*, the colour of the operculum changes from green to a reddish-yellow near the time of operculum shed, and opercula could be easily removed from the flowers, even when there was no evidence of lifting. Flowers at this stage of development were designated as at anthesis (see also Hodgson 1976a). Generally, not all flowers in the same umbel were at the same stage of development, and only buds near anthesis (determined by operculum colour and ease of removal) were emasculated. All others were removed.

Emasculation was often found to be more easily performed at just below the staminal ring in *E.nitens*. In contrast, emasculation at just below the staminal ring was exceedingly difficult in *E.globulus* ssp. *globulus* because of its larger flower.

8.2.2 Pollination

Branches containing opening buds were excised and stood in water. As flowers opened they were excised and placed on paper in a desiccator (up to 24 h). Pollen was extracted by rubbing the anthers on collection paper and then stored in gelatin capsules, encased in air-tight vials, kept at *c.* 4°C if it was to be used within a few weeks, or at -18 to -30°C if longer storage was required. At designated times, approximately 0.2 to 0.5 mg pollen was applied to stigmas using the head of a match stick.

a

b

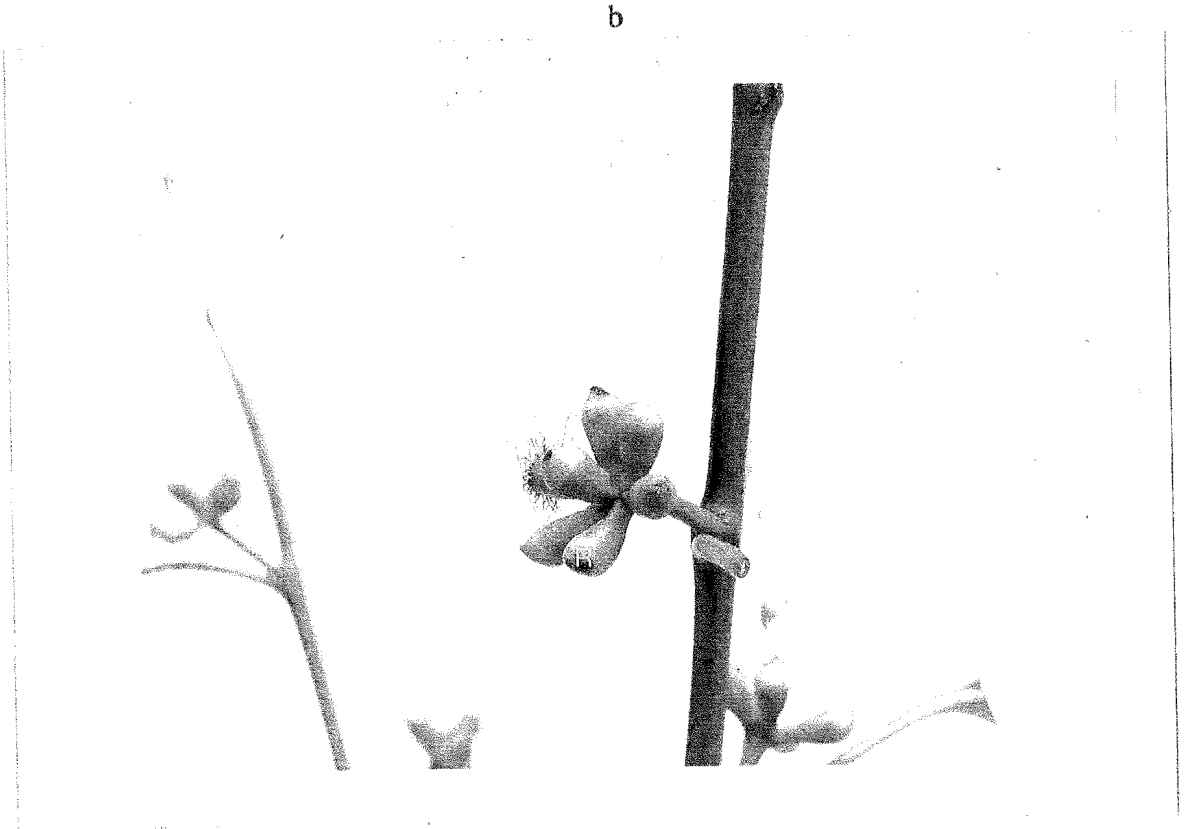


Figure 8.1 Open flowers of (a) *E.nitens* "juvenile-persistent form" and (b) *E.nitens* "early-adult form". A= angular ridge on bud, P= pointed operculum, R= rounded operculum.(X 2)

Figure 8.2 Open flower of *E.globulus* ssp. *globulus*.

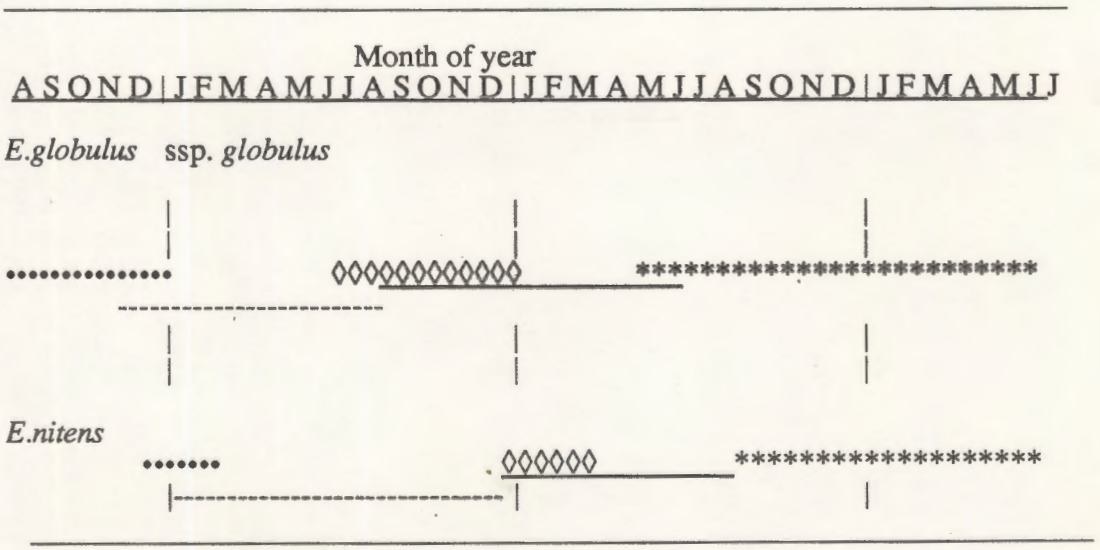


Figure 8.3 Development of flower and seed crops on *E.globulus* ssp. *globulus* and *E.nitens* (based on visual observations). Phases are, initiation of inflorescence buds (•••), flower buds (---), open flowers (◊◊◊), unripe seed(__) and ripe seed(***). After Cremer *et al.* (1978).

8.2.3 Capsule harvest

About three to five weeks following emasculation, bags were removed from branches and the number of buds remaining and their general appearance were noted. Capsules were picked approximately 12 months following pollination, e.g. October 1985 for *E.globulus* ssp. *globulus* and January for *E.nitens*. Each capsule was kept separate and dried in a glasshouse for up to two weeks. Numbers of seed per capsule were evaluated and total seed weight per capsule measured to 0.1 mg. In both *E.globulus* ssp. *globulus* and *E.nitens*, the seed is generally black and distinctly identifiable from the lighter coloured chaff.

8.2.4 Pollen germination

The viability of stored pollen was tested both *in vitro* and *in vivo*. Tests were carried out *in vitro* by applying pollen to an agar medium mounted on microscope slides. The agar medium comprised 15% sucrose, 10% Brewbaker's solution (Brewbaker and Kwack 1963) and 11% agarose. The slides were incubated at 25°C (found to give better germination than 20 or 30°C), in petri dishes lined with moist filter paper. After 48 h they were examined using a light mircoscope.

Tests were carried out *in vivo* by applying pollen to stigmas of *E.globulus* ssp.*globulus* flowers (emasculated seven days previously and isolated in the standard bag), and excising the styles 48 h later for microscopic examination. Preparation and examination of the stylar material was based on the method of Griffin *et al.* (1982a).

8.2.5 Specific crosses with *E.globulus* ssp. *globulus* maternal parent

Controlled pollinations were made from July until October 1984 on six open growing trees in the grounds of the University of Tasmania. Although *E.globulus* ssp. *globulus* occurs naturally in the immediate vicinity, the trees used were planted and the origin of their parents is unknown. Trees were chosen on the basis of readily accessible branches bearing sufficient flower buds. 1984 was a very heavy flowering year.

The timing of stigma receptivity was examined using fresh *E.globulus* ssp.*globulus* pollen applied (from a separate tree) at one of eight time intervals, viz. from four to 24 days from the time of emasculation (see Table 8.1). An average of nine buds were pollinated at each time on each of two trees. The potential for hybridization with *E.nitens* was studied on these two trees, and an additional six trees (see Table 8.2). A total of 129 buds were pollinated at various times following anthesis. The late flowering of two *E.globulus* ssp.*globulus* trees (January) and the early flowering of one *E.nitens* (November) allowed both fresh and stored *E.nitens* pollen to be used.

8.2.6 Specific crosses with *E.nitens* maternal parent

The trees used were edge trees in trial plantings, and flowered heavily in January/February 1984 and 1985. At the time of the studies they had been planted for five to 10 years. Preliminary intraspecific crosses made in 1984 on four mothers, incorporating *c.* 300 flowers, indicated that flowers needed to be pollinated about a week after anthesis to achieve reasonable capsule set (*c.* 30 to 40%). In 1985, nine trees were chosen as mothers, a total of 1004 buds were emasculated and 856 buds pollinated (some were damaged). Pollen was usually applied nine days after emasculation.

Two types of crosses were attempted. Firstly, a range of intraspecific crosses was undertaken using fresh pollen, on about 30% of the flowers. Secondly, a range of crosses with other *Eucalyptus* species was attempted on the other flower buds (fresh pollen was usually used, except for crosses involving *E.globulus* ssp. *globulus* and *E.urnigera*, where pollen had been frozen for up to 10 months). The crosses attempted are listed in Table 8.3.

8.2.7 Statistical analysis

Because of insufficient sample size in crosses with *E.globulus* ssp. *globulus* maternal parents, only the data from the crosses using *E.nitens* maternal parents were analysed. Differences in the percentage of flowers that set into capsules were analysed using the arc-sine transformation of the proportion (Sokal and Rohlf 1981) of flower buds on each umbel that set into capsules. Average seed weight was calculated as the total seed weight from a capsule divided by the number of seeds. Because of the non-orthogonality of the data, analyses were made using oneway ANOVA (Anon. 1986), comparing different pollination-types (self, open, *E.gunnii*, *E.nitens* (outcross) and other *Eucalyptus* species) within a mother and different mothers for a given pollination-type.

8.3 Results

8.3.1. Capsule set and seed yield in *E.globulus* ssp. *globulus*

The influence of time from emasculation to pollination on capsule set, is detailed in Table 8.1. Pollinating flowers which were emasculated before anthesis resulted in much lower capsule set than buds which were at anthesis. The pattern of capsule set with time from emasculation to pollination, for emasculations at anthesis, was similar for both trees, viz., little or no capsules were set if pollen was applied 12 or more days after anthesis, some capsules set even if pollination was only four days after anthesis, and 50% or more of flowers set capsule if pollinated from day four to day eight. Some indication of the relative success of pollinations could be gained at both the time of pollination and a few weeks later. Firstly, at the time of pollination, almost 50% of flowers pollinated 18 days or more after emasculation were showing signs of poor health, whereas almost none of more recently

emasculated buds appeared unhealthy (Table 8.1). Secondly, when the pollination bags were removed, none of the 19 buds on Tree 1 pollinated 16 or more days after anthesis were attached (Table 8.1). Similar trends were observed in Tree 8.

All capsules contained seeds, the number per capsule ranging from 2 to 116 with a mean of *c.* 50 seeds for Tree 1 and 28 seeds for Tree 8. There were no clear trends in the amounts of viable seed per capsule with the time of pollination. However, the means are based on an average of less than four capsules. These mean seed yields (Table 8.1) are consistently greater than those obtained from open-pollinated capsules. For instance, the mean seed yield per capsule for 10 capsules open-pollinated during 1984 are, 4 for Tree 1, and 14 for Tree 8.

Self-pollinated flowers set capsules in only one of the six trees, i.e. Tree 12, in which two of the six buds set capsules. Although some flowers were still attached on most of the other trees when the bags were removed (*c.* three weeks after the last flower opened) bud swelling was conspicuous for Trees 1 and 12 only. The buds from Tree 1 were still attached some 4 months following flowering and it is possible that these buds were vandalized, as they were located on the branch most easily reached and near a walkway. The two self-pollinated capsules from Tree 12 yielded 8 and 11 seeds. This is comparable with that from open-pollinated flowers, i.e. 14 seeds, but much less than that from two capsules from outcross controlled-pollinations which produced 57 and 67 seeds.

Despite numerous attempts, with a range of pollen freshness and parents, no flowers set when pollinated with *E.nitens* (Table 8.2).

8.3.2 Pollen germination

Stored *E.nitens* pollen (8 months at -18°C) was found to readily germinate after 48 h, both *in vitro* (Figure 8.4a) and *in vivo* (Figure 8.4b). About 50% of *E.nitens* pollen grains appeared to be germinating *in vitro*. On the two styles examined there were *c.* 500 and 900 pollen grains of which *c.* 100 and 500 had well developed pollen tubes (≥ five times pollen grain diameter, and often 50 times). Pollen tubes were on average about four times longer for *in vivo* compared with *in vitro* germination. The inability to effect capsule and seed set in the *E.globulus* ssp. *globulus* flowers pollinated with *E.nitens* (Table 8.2) would not appear to be a consequence of inviable pollen or some mechanism hindering pollen germination on the stigma.

Table 8.1 Effects of time from emasculation to pollination of flower buds on capsule set and viable seed obtained from *E.globulus* ssp. *globulus* flowers artifically pollinated.

	Time from emasculation to pollination (days)							
	4	6	8	10	12	14	16	18-24
Emasculated before anthesis†								
Tree 1								
Number								
(a) emasculated	-	7	4	7	6	-	5	7
(b) pollinated††	-	6	4	7	5	-	5	4
(c) attached *	-	4	2	4	2	-	1	0
(d) capsules**	-	0	1	1	0	-	1	0
Seed /capsule	-	-	8	36	-	-	13	-
Emasculated at anthesis								
Tree 1								
Number								
(a) emasculated	4	14	10	10	12	9	8	15
(b) pollinated	3	12	10	10	10	8	7	12
(c) attached	3	12	8	7	9	4	0	0
(d) capsules	3	8	5	2	1	0	0	0
Seed/capsule	37	52	67	41	57	-	-	-
Tree 8								
Number								
(a) emasculated	6	-	6	-	5	-	7	12
(b) pollinated	6	-	6	-	5	-	6	12
(d) capsules	1	-	3	-	0	-	0	0
Seed/capsule	24	-	30	-	-	-	-	-

†, probably at least a week before anthesis

††,(a)-(b) indicates the number of buds which had fallen or damaged styles

*, *c.* 40 days after anthesis

**, *c.* 12 months after anthesis

Table 8.2 Details of controlled crosses attempted between *E.globulus* ssp. *globulus* (maternal) and *E.nitens* (paternal).

	Maternal parent		Pollen (paternal)	
Tree No.	Days to pollination	No. flowers	Freshness*	No. fathers
1	6 - 15	8	frozen	2
8	9	9	frozen	3
15	16	4	frozen	1
11	13	10	frozen	3
12	9	11	fresh	1
18	7	7	fresh	1
19	10	33	fresh	4
21	9	47	fresh	10

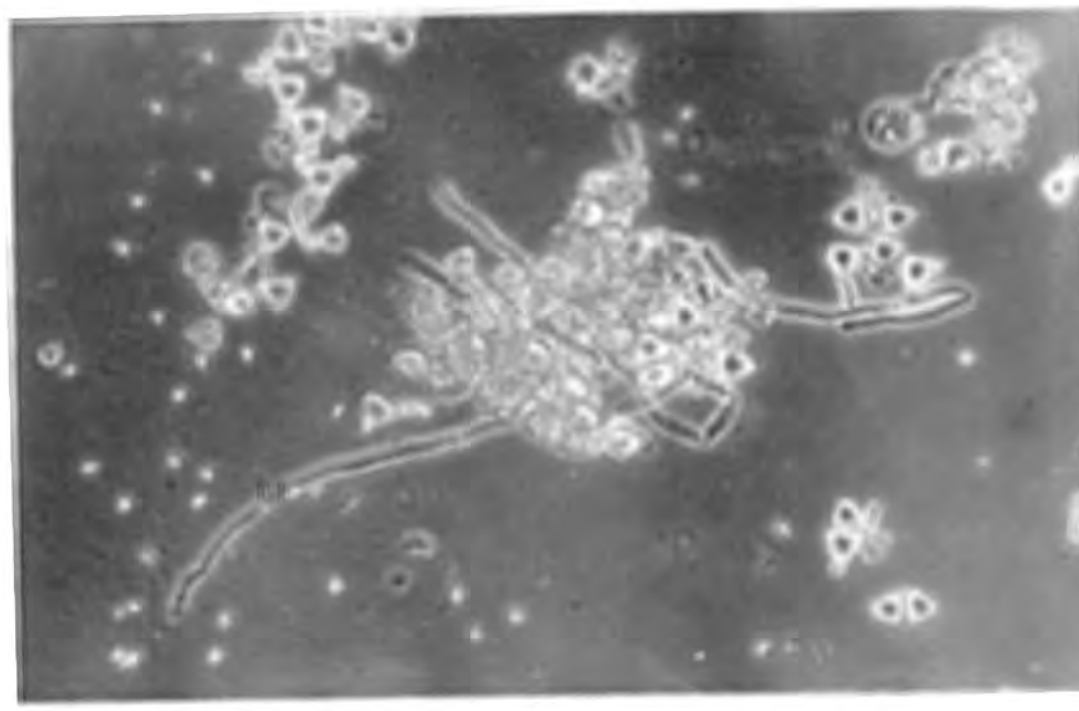
* stored at -18 to -30°C, for up to 8 months (frozen), or stored at 4°C, for up to two weeks (fresh).

Table 8.3 Details of intraspecific and interspecific controlled crosses attempted with *E.nitens* (maternal) in January and February 1985. After Potts *et al.* (1987).

		Number of			
Cross type	Crosses	Mothers	Fathers	Flowers pollinated	Capsules harvested (success)
Intraspecific (*E.nitens* x*E.nitens*)					
Self-pollinated	12	12	12	473	179 (38%)
Open-pollinated	7	7	-	347	264 (76%)
Outcross	23	5	9	272	118 (43%)
Interspecific (*E.nitens* x species)					
E.dalrympleana	1	1	1	3	2(67%)
E.globulus ssp. *globulus* †	15	8	4	153	35 (23%)
E.gunnii	17	10	4	293	106 (36%)
E.cordata	1	1	1	18	6 (33%)
E.johnstonii	2	1	2	22	15 (68%)
E.morrisbyi	2	2	1	29	13 (45%)
E.urnigera †	1	1	1	6	1 (17%)
E.viminalis	2	1	2	19	12 (63%)

†, stored pollen, otherwise fresh pollen (as Table 8.2)

a



b

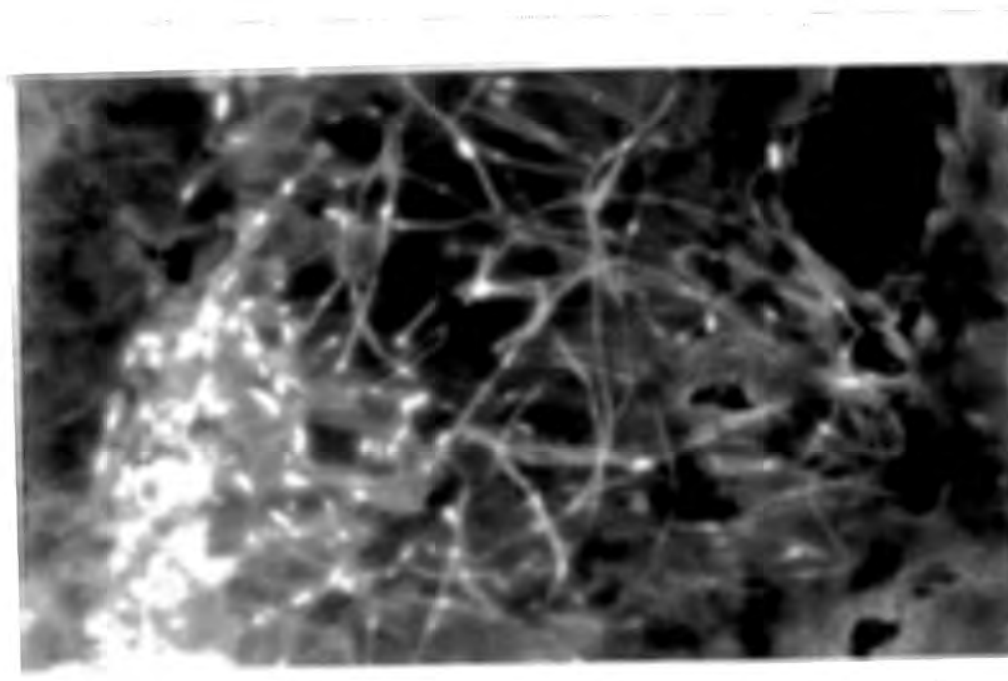


Figure 8.4 Germinating pollen grains of (a) *E.nitens in vitro* (X 350), and (b) *E.nitens in vivo,* on the stigma of *E.globulus* ssp. *globulus* (X 200). Pollen had been stored for nine months at -18°C. Pollen grains had been on an agar medium {in (a)} or on a stigma {in (b)} for 48 h, before they were prepared for examination. PT= pollen tube.

8.3.3 Capsule set and seed yield in *E.nitens*

All trees were on a level site of less than 0.1 ha, except the "early adult-form" Toorongo tree, which was *c.* 500m away. Over two consecutive flowering seasons there were consistent differences in the relative flowering times of different trees (Figure 8.5). Most trees started and completed flowering within three to four weeks. Since flowering patterns are based on a single tree for each provenance, it is not possible to determine if the differences in flowering times amongst trees reflect inherent differences amongst the provenances. However, where there were three or four trees flowering in a provenance, e.g. the "juvenile-persistent form" Toorongo provenance (a family consisting of seed from three open-pollinated trees), the range in flowering times amongst trees within a provenance was often uniform. Hence, the similarity between years, and also trees from the same provenance, suggests a strong genetic control of flowering, at least at the population level.

A number of intraspecific crosses, some reciprocal, between vastly different provenances (geographically and morphologically) were successful. In addition, all types of interspecific crosses attempted produced some capsules (Table 8.3), even though specific crosses on some trees or branches failed. Seed set was successful in crosses with *E.cordata* Labill., *E.dalrympleana, E.globulus* ssp. *globulus, E.gunnii, E.johnstonii* , *E.morrisbyi* Brett, *E.urnigera* and *E.viminalis*. Capsules still maturing from crosses made in February 1986 suggest that crosses with *E.rodwayi* Hook.f. and *E.ovata* have also been successful.

On each mother, the mean percentage of artificially pollinated flowers (self- and open-pollinated excluded) that developed into capsules ranged from 11 to 71%, with an overall mean of 39%. The percentage of flowers that developed into capsules, was significantly affected by the maternal parent, for each of the pollination types (Fig. 8.6a), e.g. self ($P<0.001$), open ($P<0.05$), *E.gunnii* ($P<0.001$) and *E.nitens* ($P<0.01$). However, for each mother, there were generally no significant differences ($P<0.01$) amongst pollination types (open-pollination excluded) in the percentage of flowers that set seed. In all instances a much higher proportion of open-pollinated flowers set seed, compared with controlled pollination flowers, viz., 76% c.f. 39% overall.

The number of seeds per capsule was significantly affected by maternal parent and pollination-type (Figure 8.6b). In both the *E.nitens* (outcross) and *E.gunnii* pollination-types, the maximum differences between mothers was *c.* 6 seeds capsule^{-1}, with the Southern N.S.W. tree (Tree 18) producing the least amounts of seed, and the Macalister (Tree 01) and Northern N.S.W. (Tree 04) trees producing the greatest amounts of seed. Differences amongst mothers, for open and self pollination-types, may reflect differences in relative activity of pollination vectors and self compatibility. However, as it was the Southern N.S.W. tree which again had the lowest number of seeds per capsule, it is possible that low amounts of seed produced by the Southern N.S.W. tree, relative to other trees, for open and self pollination-types, may indicate a real low rate of seed set. For all mothers, the mean number of seeds from self- and open-pollinated capsules were only *c.* 25 and 45%, of that

from flowers hand-pollinated with *E.nitens* and *E.gunnii* pollen respectively. This agrees with the findings for *E.globulus* ssp. *globulus* (Table 8.1). In contrast, there were no significant differences within a mother, in seed yields from intraspecific or interspecific crosses with applied pollen, e.g. overall, seed yields were *c.*8 and 7 seeds capsule^{-1}, from *E.nitens* and *E.gunnii* pollination-types respectively (Figure 8.6b). Furthermore, the average seed yields for single trees were not significantly affected (P>0.05) by a range of *E.nitens* fathers (Figure 8.7a) or by a range of species (Fig. 8.7b). On the Southern N.S.W. tree, pollen was also applied which had been scraped from emasculations (dried in a desiccator for 24 to 48 h) of the Macalister tree. Although the average seed yield from flowers crossed with this pollen {Macalister (emasculated) in Figure 8.7a} was 2.1 seed capsule^{-1} lower than that from flowers crossed with pollen extracted by standard procedures {Macalister (excised) in Figure 8.7a}, the average yields were not significantly different (P>0.05).

The mean seed weight from all capsules harvested ranged from 0.1 to 0.6 mg. Mean seed weight, within a pollination-type, was significantly (P<0.01) affected by the maternal parent, for self and open pollination-types only (Fig. 8.6c). Differences in seed weight amongst all pollination-types, within a tree, were only significant for the Southern N.S.W. tree (P<0.001). In this tree ,the self-pollinated seed was significantly different from the other pollination-types, largely due to the occurrence of a few capsules with no seed (although there was indication that fertilization had taken place, without full seed development).

The hybrid nature of progeny from the crosses has been verified (see Chapter 9).

8.4 Discussion

On the basis of capsule set (Table 8.1), *E.globulus* ssp. *globulus* stigma are receptive a few days after anthesis and remain so for up to 12 days. It appears that maximum receptivity takes place about six to eight days after anthesis. This closely corresponds to time at which stigmas first develop a sticky and somewhat flattened appearance. The association between optimal pollination times and this morphological change in stigmas, has been reported in all other detailed studies within *Eucalyptus*, viz., *E.grandis* (Hodgson 1976a,b,c) *E.regnans* (Griffin and Hand 1979) and *E.gunnii* (Cauvin 1984). Visual observations show that in *E.nitens*, stigmas undergo similar changes about seven or eight days after anthesis (near the time chosen for pollinations in this study). In both *E.globulus* and *E.nitens*, applied pollen easily fell off stigmas which were not sticky, but pollen became firmly attached to those which were sticky. The stickiness of stigmas therefore appears to be an effective mechanism for securing pollen.

In eucalypts, the length of time that stigmas remain receptive and the optimal time of pollination differs by over a week amongst species. In *E.regnans*, seed set is unlikely before eight days (from anthesis), but continues for up to 18 days (Griffin and Hand 1979). However, in both *E.grandis* (Hodgson 1976a) and *E.gunnii* (Cauvin 1984), some seed set occurs following pollination at anthesis, and maximum receptivity occurs about five days after anthesis. The similarity between *E.globulus* ssp. *globulus* and *E.nitens*, and *E.grandis* and

E.gunnii, all species of the subgenus *Symphyomyrtus* (Pryor and Johnson 1971), support the suggestion by Griffin and Hand (1979) that the floral process in eucalypts is under strong genetic control.

Visual observations found that most pollen was shed from both *E.globulus* ssp. *globulus* and *E.nitens* by about the fourth day after anthesis, i.e. a few days before stigmas reach maximum receptivity. Hence, the partially protandrous nature of these flowers would partially favour pollination from another source, though it may be from other flowers on the same tree. Self-pollination was found to occur in both species to varying degrees. Based on the percentage of self-pollinated flowers that set capsules, indications are, that some degree of self incompatibility exists within *E.globulus* ssp. *globulus* (capsules set in only one of the six trees where pollinations were attempted). In contrast, self-pollinated seed was set in 12 of the 14 *E.nitens* trees. However, the results of the 1984 and 1985 season differed markedly, for two of the three trees examined in both years, viz., 10 and 64% for Tree 4, and 0 and 12% for Tree 5. Lack of success with self-pollinations in *E.globulus* ssp. *globulus*, and more success in *E.nitens*, may also be due to problems associated with just a few large *E.globulus* ssp. *globulus* flowers in pollination bags, compared with many more smaller flowers in *E.nitens* self-pollinations. The low amounts of seed per capsule in *E.globulus* ssp. *globulus* self-pollinations, relative to artificial intraspecific pollinations (i.e. 9 c.f. 62 seeds capsule^{-1}, for only one tree), is an additional indication that some degree of self-incompatibility may exist. The low seed yield per capsule in self-pollinated *E.nitens* (only 27% of that in controlled intraspecific pollinations, and 68% of that in open-pollinations), may reflect a degree of self-incompatibility. Poor seed yields in self-pollinations, of both species, may also be a reflection of the relative amounts of pollen that reached stigmas {self-pollinations did not have pollen directly applied to the stigma, nor were any insects put in bags to aid pollination (see Eldridge and Griffin 1983)}. Indeed, reduced seed yield after manipulated selfing compared with outcrossing, has been demonstrated in *E.grandis* (Hodgson 1976a) and *E.gunnii* (Potts *et al.* 1987).

Of the differences in flowering time (Figure 8.5) and seed maturity amongst the *E.nitens* trees examined, the consistent difference between trees with the "juvenile-persistent form" and "early-adult form" is of particular interest. Seed from three trees with the "early-adult form" was not ripe in January, and did not ripen for *c.* two months thereafter, in either 1984 or 1985. In contrast, seed from all trees with the "juvenile-persistent form" was always ripe by January. Furthermore, all trees with the "early-adult form", when compared with those with the"juvenile-persistent form", were characterised by rounder flower buds (Figure 8.1b c.f. Figure 8.1a) carried on longer peduncles, and capsules with more exsert valves. Measurements made from 85 herbaria collections (Table 8.4) suggest that these aspects of floral morphology are consistently different between the two forms.

Eucalypts, like many plant genera, exhibit successful hybridization between pairs of species (Pryor 1976). Pryor and Johnson (1971) state that the frequency of hybridization reflects the hierarchy of taxonomic affinities. Interspecific hybrids occur in natural forests (Pryor 1976; Potts and Reid 1983,1985), though extensive genetic mixing is largely inhibited and species breakdown avoided (Pryor 1976). However, interspecific hybridization often

becomes extensive overseas (Pilipenka 1969), where geographic and temporal isolation barriers are removed, when many species are planted together. It is overseas that large interspecific hybridization programmes have been undertaken, successfully producing many combinations, e.g. Russia (Pilipenka 1969) and France (Potts and Potts 1986; Potts *et al.* 1987). Whilst the present study found that *E.nitens* (maternal) could be readily hybridized with all other species tested (Table 8.3), it must be emphasised that all species are in the same series (*Viminales*), and hence relatively close taxonomically. Capsules still maturing from pollinations in February 1986, contain two inter-sectional crosses (*E.ovata* and *E.rodwayi*) and represent species pairs more distant than the previously successful crosses. Crosses between species with wider taxonomic affinity were not attempted.

The unequal success between reciprocal combinations of *E.globulus* and *E.nitens* (Tables 8.2 and 8.3), yet the successful germination of pollen *in vivo* (Figure 8.3b) indicates some functional mechanism hindering fertilization. One possibility is that the vast differences in style length (*c.* 12 mm *E.globulus* ssp. *globulus* and 3 mm *E.nitens*), and pollen size (*E.globulus* ssp. *globulus* pollen *c.* 50 to 100% larger diameter than *E.nitens* pollen) between the two species, as initially proposed by Pryor (1956) following failure of some reciprocal crosses. Unequal success between species with different floral morphology have also been reported for *E.pulverulenta X E.caesia* (Pryor 1956), *E.globulus* X *E.viminalis* (Pryor 1976), and *E.caesia* X *E.sideroxylon* (Beardsell *et al.* 1979). Additional evidence is provided by the failure of *c.* 30 visually receptive *E.urnigera* flower buds on two trees to set capsules when pollinated with *E.nitens* pollen, yet (limited) success of the reciprocal (Table 8.3). Successful reciprocal crosses have been made between *E.nitens* and *E.gunnii* (which is closely related to *E.urnigera*). Style lengths are *c.* 10 and 6 mm in *E.urnigera* and *E.gunnii* respectively.

The findings of this study demonstrate some salient implications for eucalypt breeding programmes. Artificial pollinations can produce a range of intraspecific and interspecific hybrids, as has been shown elsewhere for other species within the major commercial subgenus, *Symphyomyrtus*. The relative merits of such hybridization is not addressed here. Across all successful combinations, the percentage of flowers that developed into capsules, when artificially pollinated at a receptive stage, averaged 42% in *E.globulus* and, 43 and 35% in *E.nitens*, for intraspecific and interspecific combinations respectively. These compare favourably with an average set of fruit from all successful interspecific combinations in Russia of 43% (Pilipenka 1969), averages of 40 to 50% for a range of crosses on *E.gunnii* in France (Cauvin 1984) ,and averages of 50% at near optimum pollination times for intraspecific crosses in *E.regnans* (Griffin and Hand 1979). Though breeders are more likely to be interested in the number of plants per flower crossed (see Potts *et al.* 1987), those undertaking controlled pollinations with species of *Eucalyptus* (in the absence of data on seed yields and viability) could work on 40 to 50 capsules per 100 flowers. Seed yields will vary with the maternal parent more so than the paternal parent (see Table 8.1 and Figures 8.5b and 8.7b), but on average will be at least twice that from open-pollinations in both *E.globulus* ssp. *globulus* and *E.nitens*.

Since there are considerable differences in flowering times of individual trees, and self-fertility exists to some extent, courses of action should be taken to minimise the extent of inbreeding in seed orchards ,and associated depressed growth in progeny (see Hodgson 1976c; Eldridge and Griffin 1983; Potts *et al.* 1987). Where there are significant differences in flowering times of different provenances, it may be necessary to plant separate areas of seed orchard with each provenance. In cases where crossings between provenances and/or species with different flowering times are desirable, controlled pollinations may need to be made. Pollen from early or late flowering individuals, or species flowering in a different season, can be adequately stored (at sub-zero temperatures) until required.

In species such as *E.globulus* ssp. *globulus* and *E.nitens*, where the first flowers appear about five years after sowing, and viable seed can be obtained about 12 months after flowering, a generation could be completed within six years. Where interspecific crosses are being made, species with more rapid seed maturity should be chosen as mothers, other things being equal, e.g. *E.nitens* X *E.gunnii* takes one year to mature on *E.nitens* mothers, whilst the reciprocal takes two years. It may be possible to obtain viable seed a little earlier, by using earlier flowering species, in some interspecific crossing programmes, and/or by using grafted seed orchards (Eldridge 1978).

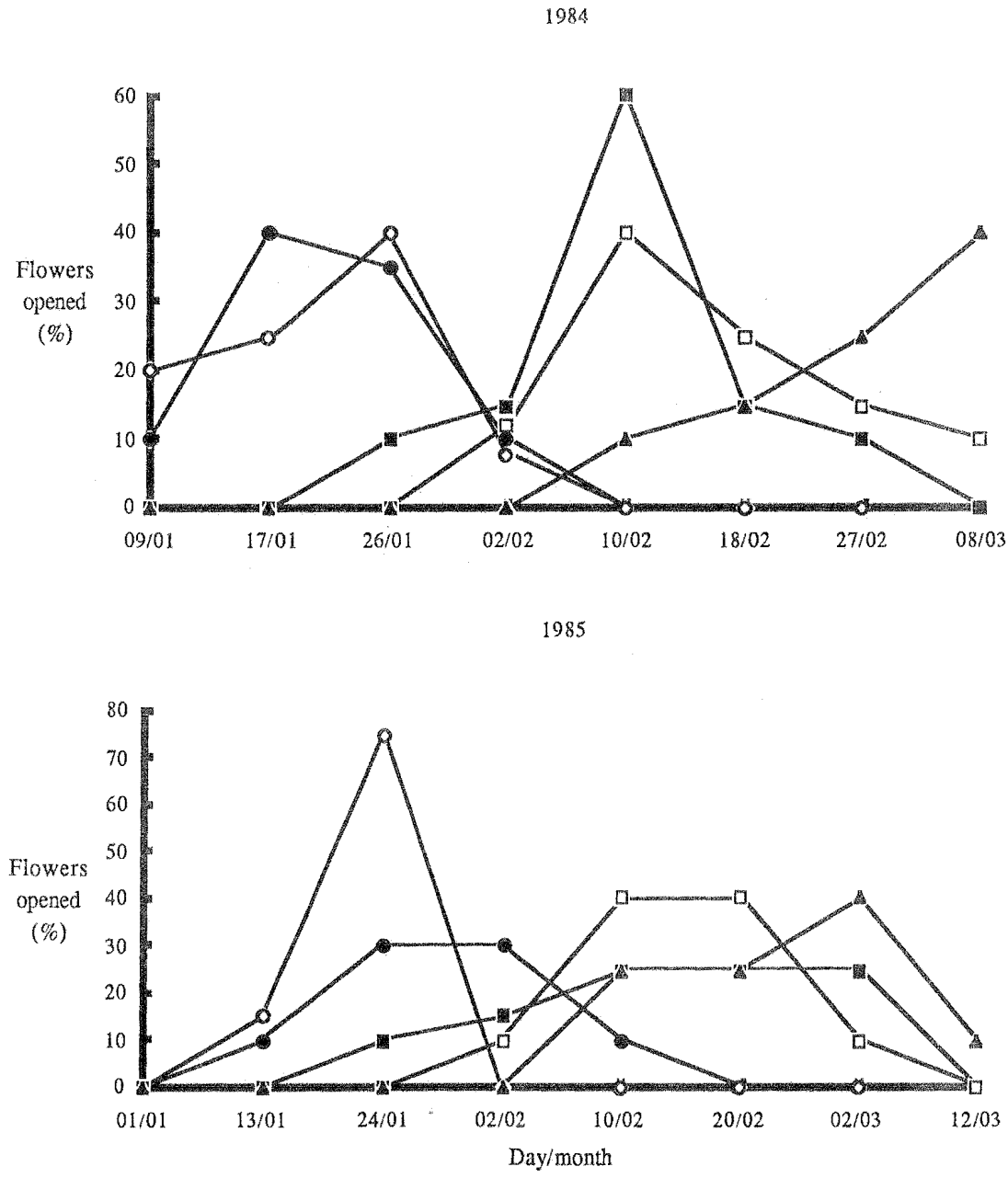


Figure 8.5 Flowering patterns in 1984 and 1985 for five *E.nitens* trees at Myrtle Bank, Tasmania. Points indicate the percentage of flowers that had opened over the previous seven days. Trees are from the following provenances:- (●) Toorongo with the "juvenile-persistent form", Tree 05; (▲) Toorongo of the "early-adult form", Tree 08; (□) Macalister, Tree 01; (○) Southern N.S.W., Tree 18; and (■) Northern N.S.W. Tree 04.

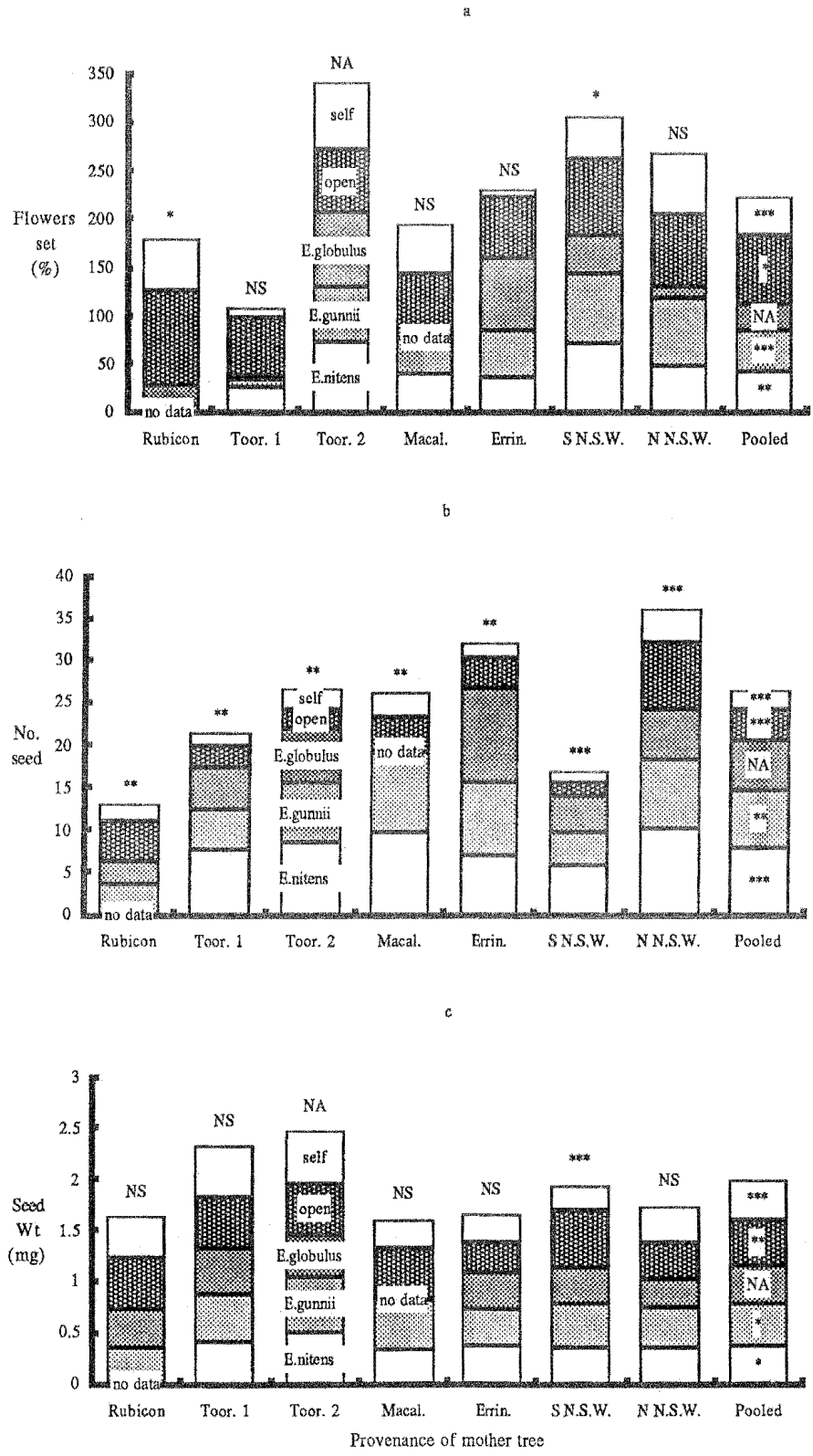


Figure 8.6 Relationships between (a) the percentage of flowers which set seed (shown as cumulative, maximum=500%), (b) mean number of seeds per capsule, and (c) mean seed weight, for five cross-types, and for trees from various provenances of *E.nitens*. The *E.globulus* cross-type failed in Macal., whilst the *E.nitens* cross-type was not attempted in Rubic. The trees in the provenances are:- Tree 12, Rubicon (probably from Southern N.S.W., rather than its labelled identity); Tree 05, Toor. 1; Tree 15, Toor. 2; Tree 01, Macal.; Tree 21, Errin.; Tree 18, S N.S.W.; and, Tree 04, N N.S.W. Levels of significance, for comparisons within a tree (amongst cross-types) are shown at the top of each bar, whilst those within a cross-type (amongst trees) are shown in the corresponding section of the pooled bar:- are, NA (not assessed, insufficient data), NS (P>0.05), P<0.05 (*), P<0.01 (**) and P<0.001 (***).

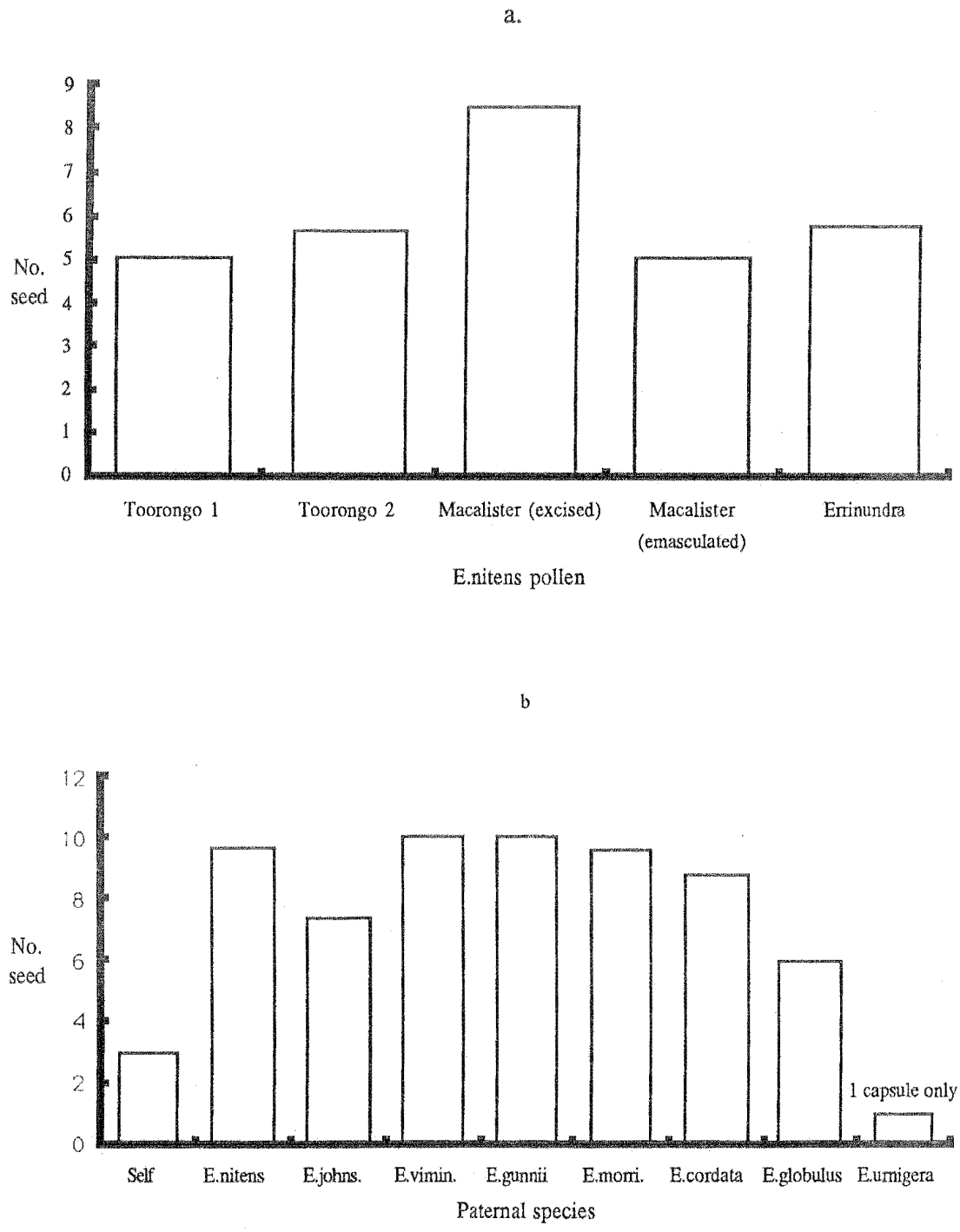


Figure 8.7 Average numbers of seeds per capsule from (a) intraspecific crosses on Tree 18 (Southern N.S.W.), and (b) all crosses on Tree 1 (Macalister provenance), apart from the *E.globulus* ssp. *globulus* crosses , which were on Tree 4 (Northern N.S.W. provenance).

Table 8.4 Flower bud shape and capsule measurements from *E.nitens* herbaria collections.

Provenance[A]	Nº individuals[B]	Peduncle length (mean ± S.D.,mm)	Valve level[C] (mm)	Bud shape[D]
"juvenile-persistent form"				
Rubicon	7	7.6 ± 1.2	0.1	angular
Toorongo	7	6.4 ± 1.1	0.0	angular
Macalister	4	8.3 ± 1.7	0.3	angular
Southern N.S.W.	19	7.0 ± 1.6	0.1	angular
Northern N.S.W.	29	7.4 ± 1.6	0.1	angular
"early-adult form"[A]				
Toorongo	2	11.3 ± 1.1	-	rounded
Errinundra	17	9.9 ± 1.8	0.8	rounded

A, see Pederick (1979)

B, average of four measures per individual

C, distance valves protruding above disc rim (valves below rim scored as 0)

D, angular (see Figure 8.1a) and rounded (see Figure 8.1b)

CHAPTER 9

GERMINATION AND MORPHOLOGY OF CONTROLLED CROSSES

9.1 Introduction

E.nitens is a fast growing species under plantation conditions, and breeding programmes may be implemented for the improvement of desirable traits, such as frost resistance. In the previous chapter, it was demonstrated that successful seed set was achieved in a number of controlled intraspecific and interspecific crosses using *E.nitens* maternal parents. Similarly, effective seed set has been reported in other eucalypt breeding programmes (e.g. Pilipenka 1969; Hodgson 1976b; Potts and Potts 1986). However, successful controlled breeding studies are not only influenced by the ability to set seed, but also by the subsequent germination and growth of the progeny. Potts *et al.* (1987) found that the low proportion of plants raised per flower pollinated in specific crosses onto *E.gunnii* mothers was due to a combination of low percentage capsule set, low seed yield, poor germination and low survival of individuals. Hodgson (1976b) recognised 15 types of abnormality in progeny of self pollinated *E.grandis*, of which 10 appeared detrimental. Clearly, the matters of germination and growth of progeny in breeding programmes need to be adequately understood.

Other interesting aspects in progeny from breeding programmes are those concerning the inheritance of various characters. Whilst there are much data available concerning the inheritance of morphological characters in *Eucalyptus* species, most data have been derived from open-pollinated progeny on and around naturally occurring hybrids (Pryor 1957b; Potts and Reid 1983, 1985). Some controlled crosses have been made in Australia (Brett 1949 cited by Pryor 1951; Pryor 1951,1954,1956,1976; Griffin and Hand 1979; Eldridge and Griffin 1983) though the comparative morphological features of the hybrids have not always been reported. In some overseas breeding programmes, the morphological features of F_1, and even some F_2 hybrids, have been documented (Pilipenka 1969; Cauvin *et al.* 1987). The production of a number of interspecific hybrids in this study enables more valuable morphological data to be collected from hybrids. In addition, the production of a number of families of the same hybrid type (e.g. *E.nitens* X *E.gunnii*) permits examination in finer detail of any patterns which may exist within interspecific hybrids.

Apart from the work of Hodgson (1976a,b,c,) there is a distinctive lack of information on the growth and morphology of controlled crosses within a single *Eucalyptus* species. The controlled intraspecific crosses within *E.nitens* present a unique opportunity

to study the growth of progeny and inheritance of characters, since there is a considerable amount of natural variation within *E.nitens* (Pederick 1979; Pederick and Lennox 1979).

The purpose of this chapter was twofold. Firstly, the hybrid nature of both the interspecific and intraspecific crosses (at least those from parents of widely differing provenances) needed to be assessed to validate the results of controlled pollination studies (Chapter 8). The second purpose was to assess the germination of seed and growth of progeny produced from controlled crosses, paying particular attention to aspects of seed viability and the development of any abnormality in progeny.

9.2 Materials and Methods

9.2.1 Seedling growth and characters assessed

Seed was sown onto moist vermiculite in punnets and germinated under controlled conditions (22°C day/17°C night, 14 h photoperiod). Sheets of glass were placed over punnets to reduce the likelihood of seed movement between punnets. Seed was scored as germinating when the seed coat had been visibly ruptured by the radicle. This definition is similar to that of Bachelard (1967). Germination counts were made every two days until 30 days after sowing. Seedlings were transplanted into polythene pots and grown in a heated glasshouse as described in Section 4.2.

Approximately four months after transplanting, seedlings were assessed for the ordered multistate and numerical characters listed in Table 9.1. Multistate characters were scored in comparison with standards. At the time of scoring, leaves at the eighth node were not fully expanded on about 5% of the seedlings and leaf characters were assessed from a leaf at the sixth or seventh, rather than the usual eighth, node. Two *E.nitens* intraspecific crosses (Tree 01 X Tree 21 and its reciprocal) showed extremely slow growth and were not assessed for characters until eight months after transplanting.

9.2.2 Statistical methods

Statistical comparisons of seed germination parameters and survival amongst cross types and maternal parents were generally not made because of the low number of replications in many treatments and lack of orthogonality in design. Analyses of 18 morphological characters (Table 9.1, with no transformations, and excluding Ht, NE, NL, SD1 and SD2) were performed by multiple discriminate function analysis (Fisher 1936) with the aid of the Statistical Package for the Social Sciences (Anon. 1986). This has been widely used in hybridization studies with *Eucalyptus* species, e.g. Clifford and Binet (1954), and Potts and Reid (1983). Oneway analyses of variance were also performed on each character used in the discriminate function analyses (Tables 9.3, 9.4 and 9.5).

9.3 Results

9.3.1 Germination and growth

On average 82% of all seed sown germinated, with 50% germination occurring about 10 days after sowing (Table 9.2). Seed from all cross types proved viable with 100% germination in 25 of the 86 seedlots sown. The single seed obtained from the *E.nitens* X *E.urnigera* cross had failed to germinate even eight weeks after sowing, although it appeared fully imbibed. Squash tests were not employed to assess viable ungerminated seeds, since the seeds were considered too valuable to risk loosing any potential plants, and hence germinative capacity, as used by Grose (1963), could not be evaluated.

There appear to be a number of distinct differences in germination parameters and seedling vigour between various cross types. Of particular interest in the intraspecific *E.nitens* crosses, is the higher percentage seed germination, and lower percentage seedling mortality and seedling abnormality, in outcrosses compared to open-pollinated and self-pollinated crosses (Table 9.2). There were little differences amongst these three treatments in the speed of germination as assessed by the time to 50% germination or germinative energy index (GEI, to day 14).

Of the interspecific crosses and other *Eucalyptus* species seedlots (paternal controls), only the open-pollinated *E.johnstonii* and *E.nitens* X *E.globulus* were characterized by germination percentages much lower than average. This appeared to be largely due to the immature nature of seed in some families of each seed type, e.g. the only capsules available on one *E.johnstonii* tree were barely ripe and the seed from the *E.nitens* X *E.globulus* cross on Tree 21 ("early-adult form") was unripe when harvested. There was a great range in rate of germination amongst individual seedlots, e.g. time to 50% germination ranged from 3 days to 20 days. However, there appeared to be no major differences between cross types with more than three replications (Table 9.2).

The frequency of abnormal seedlings was almost twice the average in the open-pollinated *E.morrisbyi*, open-pollinated *E.dalrympleana* and *E.nitens* X *E.johnstonii*, and over three times the average in *E.nitens* X *E.gunnii* (Table 9.2). Generally, the abnormal seedlings were characterized by very short internodes and small leaves and were termed "runts" (Figure 9.3). The abnormal *E.nitens* X *E.johnstonii* seedlings were not runts but had distorted leaves and multiple stems.

Above average mortality was recorded in three interspecific crosses and open-pollinated *E.cordata* (Table 9.2), with death generally occurring in small seedlings before expansion of the third leaf pair. Although the high mortality in the *E.nitens* X *E.gunnii* cross was associated with the death of many runts, death of runts generally contributed little to overall mortalities. However, seedlings of *E.nitens* X *E.morrisbyi* died when they were 30 cm or more tall. The pattern of death in this seedlot was characterized by wilt of the leading shoot and laterals, followed by stem darkening and necrosis. This pattern occurred on two separate occasions, viz., firstly, to about 10 seedlings growing in the

glasshouse, and subsequently to six seedlings transferred back to the glasshouse after they had been grown under artificial hardening conditions (see Chapter 10). Plating of agar with material from affected seedlings revealed that *Botrytis* spp. were associated with the mortality (A.K.Mills pers. comm.). Tall seedlings from no other families displayed this pattern of damage.

9.3.2 Morphology of controlled crosses

All six interspecific hybrids were intermediate with respect to their parental controls, as determined by separate discriminant function analyses (Figures 9.4 and 9.5). Even for parental species with relatively similar morphology, such as *E.nitens* and *E.globulus* (Figure 9.5a) the hybrids were clearly separated from each parent. In all cases, and most noticeably in *E.nitens* X *E.morrisbyi* and *E.nitens* X *E.viminalis*, the hybrid had closer affinities to the non-*E.nitens* parent (Figures 9.4 and 9.5b respectively). This may be because, in the cross involving *E.nitens* and *E.morrisbyi*, the characters SV, RO, RT and NLL were highly weighted in the discriminant function, and of these the mean value of SV and RT in the hybrids were very close to that of *E.morrisbyi* (Table 9.4). In the cross between *E.nitens* and *E.viminalis*, the characters FL, RL, SD3,RO and NLL were highly weighted in the discriminant function, and of these the mean value of SV and RT in the hybrids were similar to that of *E.viminalis* (Table 9.3). In many cases hybrids and parents were able to be separated on the basis of single univariate characters. On average for the six hybrids, about 90% of the univariate characters varied significantly ($P<0.05$) among parents and hybrids (Tables 9.3 and 9.4). Of these, the mean values for hybrids were intermediate with respect to parental means in 72% of the cases.

Intraspecific crosses were also generally distinctively different from parental controls (Figure 9.6). In the case of Tree 04 X Tree 21 and its reciprocal, one parent, Tree 21, had some overlap with the hybrids (Figure 9.6c), whilst the other parent (Tree 04) was clearly separated. In the three instances with reciprocal crosses (Figure 9.6a,b,c) the mean values for the reciprocal crosses were very similar. In three of the four analyses, the second discriminant function accounted for 16% or more of the variation (at least twice the variation as was noted in the interspecific crosses). On average, only 50% of the univariate characters varied significantly ($P<0.05$) amongst hybrids and parental controls (Table 9.5), where almost 90% varied significantly in interspecific hybrids and parental controls (Tables 9.4 and 9.5). Of those that varied significantly, the mean values for hybrids were intermediate with respect to parental means in about 60% of the cases, excluding the crosses Tree 01 X Tree 21 and its reciprocal. These crosses (Tree 01 X Tree 21 and Tree 21 X Tree 01) were exceptional, since the mean values for the hybrids were outside the range between parental means, for 11 of the 13 characters where significant differences were noted. These seedlings were those noted in Section 9.2.1 that were slow growing and had to be assessed much later. However, the differences in their mean values are not due to a different sampling date, since both assessments made on some smaller seedlings in families previously assessed agreed with family trends, and seedlings rechecked at the same time matched previous assessments.

They represent crosses involving trees derived from geographically isolated and morphologically distinct (Pederick 1979) populations (i.e. Tree 01 from Macalister provenance and Tree 21 from Errinundra provenance).

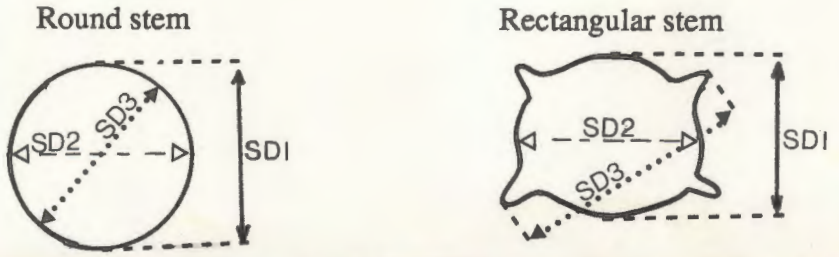
Table 9.1 Description of characters scored from seedlings.

Character	Description
	Numerical
LL*	Length of leaf lamina (mm)
LW*	Maximum width of leaf lamina (mm)
LWP*	Length from lamina base to widest point (mm)
VA*	Angle of secondary vein to midrib (°)
AA*	Angle of leaf apex (°). This was measured at the arc formed between each leaf margin at a distance of 1 cm from the leaf tip.
Ht	Seedling height (cm) three months after planting
Ht8	Height to node eight (cm), with cotyledons scored as node zero
FL	Length of terminal leaf pair (mm) fused by their margins around the apical bud. This character is pronounced in *E.nitens* where the entire leaf margin is fused even for leaves up to 80 mm in length.
NE	Number of leaf pairs expanded (length ≥ 40% LL)
NL	Number of laterals
RL	Proportion of laterals, i.e. NL ÷ (NE X 2)
LLL	Length of longest lateral (cm)
NLL	Node of longest lateral
SD1-3	Three stem diameter measures at eigth internode (mm)**
RT	Stem rectangularness, i.e. SD1/SD2
RO	Stem roundness, i.e. SD1/SD3
	Ordered multistate
LG	Leaf glaucousness (1-5, *E.johnstonii* =1, *E.gunnii* =5)
SG	Stem glaucousness (1-5, *E.johnstonii* =1, *E.gunnii* =5)
SV	Stem verrucae (1-4, *E.globulus* =1, *E.cordata* =4)
SR	Stem redness (1-3, *E.globulus* =1, *E.viminalis* =3)
SW	Waviness of wings on square stems (1-3; 1=absent, 3=maximum)†

*, from a single leaf from the eighth node

**, see Figure 9.1

†, see Figure 9.2



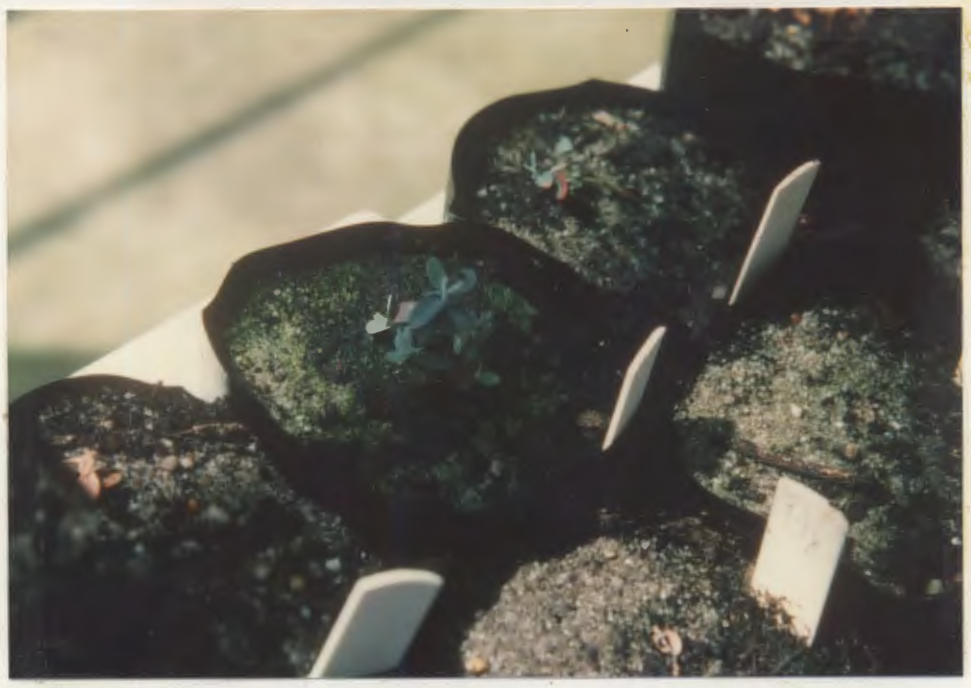


Figure 9.1 (top) cross-sections of stems showing diameters measured on seedlings. In round stems SD1=SD2=SD3 (generally) and in rectangular or square stems SD1≤SD2=SD3.
Figure 9.2 (middle) The order multistate character "stem waviness" (SW) on a *E.nitens* seedling (score=3, maximum).
Figure 9.3 (bottom) A typical *E.nitens* X *gunnii* seedling termed a "runt". This seedling had been growing for eight months under glasshouse conditions. Pot diameter = 6 cm.

Table 9.2 Some germination and growth parameters for seed from controlled crosses. Generally 16 seeds were sown in each replication.

Seed type	No of Replications	Germination %	Days to 50%	GEI*	Percentage Abnormality	Mortality
E.nitens O.P.†	15	78.7	11	.65	8.8	5.0
E.cordata O.P.	1	100.0	11	.67	0.0	18.8
E.dalrympleana O.P.	1	79.0	9	.76	10.5	5.2
E.globulus O.P.	3	84.0	5	.72	0.0	7.5
E.gunnii O.P	5	83.8	12	.66	3.6	1.7
E.johnstonii O.P.	2	69.7	14	.74	0.0	5.1
E.morrisbyi O.P.	2	90.5	9	.73	15.4	0.0
E.viminalis O.P.	3	95.3	7	.80	2.1	8.3
E.nitens intraspecific hybrids (± S.D.)						
Self pollinated	12	74.1±26.2	9±4.9	.67±.13	6.4±12	20.0±28
Outcrossed	25	87.9±12.8	11±5.8	.65±.13	1.1±3	4.4±11
Interspecific hybrids with *E.nitens* mothers						
X *E.cordata*	1	88.0	11	.51	0.0	0.0
X *E.dalrympleana*	1	100.0	9	.81	7.7	7.7
X *E.globulus*	10	61.7	9	.59	1.5	19.6
X *E.gunnii*	14	86.1	10	.72	19.5	11.4
X *E.johnstonii*	2	94.5	9	.70	9.4	3.1
X *E.morrisbyi*	2	100.0	8	.73	0.0	12.5
X *E.viminalis*	2	100.0	8	.68	3	6
Overall mean (± S.D.)		81.9 ± 19.4	10 ± 5.8	.68 ± .13	6.1 ± 13	9.2±15

*, Germinative energy index (see Bartlett 1937)

†, open-pollinated

Table 9.3 Mean univariate characters for *E.nitens*, Tree 01 (maternal), and paternal (other species) parents, and their hybrids for various interspecific hybrid types. Abbreviations for characters are as in Table 9.1. Species abbreviations are *E.cord., E.cordata* ; *E.johnst., E.johnstonii*; *E.vimin. E.viminalis*. Levels of significance from oneway-analysis (between each hybrid and its two parental controls) are, a (P>0.05), b (P<0.05), c (P<0.01), d (P<0.001), e (P<0.0001).

Character	*E.nitens*	*E.nitens* X *E.cord.*	*E.cord.*	*E.nitens* X *E.johnst.*	*E.johnst.*	*E.nitens* X *E.vimin.*	*E.vimin.*
LL	63.7	41.6e	32.0	39.1e	33.1	59.3c	51.2
LW	46.0	33.4e	33.4	33.4e	23.7	38.8e	25.6
LWP	19.6	11.7e	10.5	12.8d	15.7	14.9e	13.5
LG	2.9	3.4e	4.0	1.8e	1.0	2.8a	2.7
SG	2.7	3.1c	2.4	1.5e	1.0	2.2e	1.8
SV	1.0	2.8e	4.0	2.2e	3.1	2.5e	3.3
SR	1.4	2.2c	1.6	1.6a	1.2	2.3e	2.4
SW	1.4	1.1c	1.1	1.0b	1.0	1.0e	1.0
Ht8	26.6	24.3b	23.4	21.3c	24.1	29.8c	26.6
Ht	37.3	40.5a	40.7	33.9d	28.2	51.6e	54.2
NE	10.1	12.4a	11.9	12.5c	8.7	11.2c	12.4
FL	2.1	0.8e	0.4	0.8e	0.3	1.4e	1.2
NL	7.0	18.2e	14.5	13.6c	10.8	14.7e	16.3
LLL	14.4	14.3a	17.6	13.5a	12.4	16.5a	17.2
NLL	2.4	4.1c	4.4	3.8a	3.3	5.6e	5.7
SD3	4.5	2.8e	2.1	3.1e	1.9	3.1e	2.3
VA	72.8	59.7e	58.6	62.1e	37.9	56.1e	36.2
AA	92.3	85.6e	116.8	100.0c	118.8	73.8e	68.2
RL	0.36	0.77e	0.61	0.58c	0.62	0.67e	0.64
RT	0.90	1.00e	1.00	0.94a	0.95	0.95c	0.98
RO	0.68	0.80e	0.99	0.74c	0.77	0.80e	0.97
Nº sign.†	-	18	-	17	-	19	-
Nº inter.††	-	9	-	12	-	16	-
Nº plants	16	18	13	12	10	29	27

†, number of characters where one-way analysis was significant (P<0.05)

††, number of characters where mean value of hybrid was approximately intermediate

Table 9.4 Mean univariate characters for *E.nitens* (maternal parents), and *E.globulus* (*E.glo.*), *E.gunnii* (*E.gun.*) and *E.morrisbyi*; (*E.mor.*) (paternal parents), and their hybrids. Abbreviations for characters are as in Table 9.1. Levels of significance from oneway-analysis (between each hybrid and its two parental controls) are, a (P>0.05), b (P<0.05), c (P<0.01), d (P<0.001), e (P<0.0001).

	E.nit. (3)*	Hybrid (3)	*E.glo.* (2)	*E.nit.* (6)	Hybrid (6)	*E.gun.* (1)	*E.nit.* (1)	Hybrid (1)	*E.mor.* (1)
LL	77.5	86.6e	96.9	76.7	40.5e	19.6	76.9	47.9e	22.0
LW	51.0	41.7a	46.6	47.7	38.7e	28.5	49.4	43.4e	23.4
LWP	25.9	25.8e	33.5	23.7	12.5e	6.4	26.8	11.8e	6.5
LG	2.9	4.0e	4.8	2.8	4.0e	4.9	3.1	3.8e	4.1
SG	3.2	3.8e	4.9	2.9	3.8e	4.9	3.6	4.0a	4.0
SV	1.0	1.1d	1.4	1.0	2.7e	3.6	1.0	3.0e	3.3
SR	1.2	1.1a	1.3	1.2	1.9e	1.9	1.2	1.8c	1.6
SW	1.6	1.3c	1.0	1.6	1.0e	1.0	1.6	1.0c	1.0
Ht8	23.8	26.4e	33.7	24.8	23.9e	18.0	22.8	22.7e	14.7
Ht	33.2	44.8e	70.9	35.6	35.4d	33.7	31.6	40.8c	30.4
NE	9.2	12.3e	14.8	9.9	12.8e	15.2	9.3	11.5e	11.8
FL	3.5	1.8e	0.9	3.0	0.6e	0.1	3.9	0.8e	0.2
NL	4.1	5.4e	10.3	4.4	12.7e	15.7	6.9	14.7e	18.1
LLL	7.6	12.7e	20.7	8.8	13.4d	13.2	11.0	18.0d	12.4
NLL	1.2	3.4e	4.0	1.7	3.7e	4.8	1.8	3.4c	5.6
SD3	4.9	5.8c	5.8	4.9	3.0e	3.4	5.4	3.0e	1.7
VA	68.2	64.0a	69.5	66.2	64.6c	57.7	66.2	68.0d	55.1
AA	107.5	83.7e	80.3	92.7	135.8e	183.6	114.4	128.8e	162.7
RL	0.22	0.22b	0.35	0.24	0.51e	0.49	0.38	0.63e	0.68
RT	0.86	0.81a	0.80	0.86	0.97e	1.00	0.82	0.95e	1.00
RO	0.68	0.65a	0.68	0.68	0.79e	1.00	0.65	0.80e	1.00
Nº sign.†	-	16	-	-	21		-	20	-
Nº inter.††	-	9	-		12		-	16	-
Nº plants	32	22	23	67	71	18	16	12	7

*, number of families of the parental or hybrid type

†, number of characters where one-way analysis was significant (P<0.05)

††, number of characters where mean value of hybrid was approximately intermediate

Table 9.5 Mean univariate characters for intraspecific *E.nitens* hybrids and their parents. Abbreviations for characters are as in Table 9.1. Levels of significance from oneway analysis (between each hybrid and its two parental controls) are, a (P>0.05), b (P<0.05), c (P<0.01), d (P<0.001), e (P<0.0001).

	E.nitens Tree numbers (maternal X paternal)							
Character	18X18	18X01	01X01	01X04 04X01*	04X04	04X21 21X04*	21O.P.	01X21 21X01*
LL	88.0	78.3e	63.7	78.7e	76.9	83.0a	83.3	32.1e
LW	43.1	43.5a	46.0	49.0a	49.4	46.0a	44.2	21.7e
LWP	26.8	22.6b	19.6	22.9c	26.8	23.1a	25.2	8.8e
LG	2.2	2.6b	2.9	3.0a	3.1	2.9b	2.7	2.6a
SG	3.0	2.3b	2.7	3.2e	3.6	3.0e	2.7	2.6a
SV	1.0	1.1a	1.0	1.0a	1.0	1.1a	1.0	1.3b
SR	1.8	1.7a	1.4	1.4a	1.2	1.5a	1.6	1.4a
SW	3.0	2.5e	1.4	1.6a	1.6	2.2a	1.9	1.2a
Ht8	16.8	22.6e	26.6	26.0a	22.8	25.8a	26.3	12.8e
Ht	25.8	43.1e	37.3	38.2b	31.6	41.9c	41.1	27.2c
NE	10.0	14.2e	10.1	10.5a	9.3	11.4b	10.9	13.2c
FL	3.9	2.9c	2.1	2.9e	3.9	2.7d	2.2	1.3e
NL	3.5	6.2a	7.0	6.9a	6.9	5.1a	6.1	5.5a
LLL	7.0	13.9a	14.4	14.5a	11.0	10.0a	11.9	8.6b
NLL	2.2	1.9a	2.4	2.0a	1.8	3.3a	1.7	4.3b
SD3	4.6	5.8e	4.5	5.5d	5.4	5.6a	5.3	2.8e
VA	58.0	61.8c	72.8	69.5b	66.2	66.8a	63.6	65.2c
AA	72.5	73.6d	92.3	94.0e	114.0	79.6e	81.1	86.6a
RL	0.18	0.22a	0.36	0.36a	0.38	0.24b	0.33	0.19b
RT	0.94	0.88a	0.90	0.88a	0.82	0.88b	0.92	0.88a
RO	0.75	0.70a	0.68	0.69a	0.65	0.70c	0.75	0.76b
Nº sign.†	-	12	-	8	-	9	-	14
Nº inter.††	-	7	-	5	-	6	-	1
Nº plants	4	14	16	21	16	31	7	25

*, data from reciprocal crosses combined

†, number of characters where one-way analysis was significant (P<0.05)

††, number of characters where mean value of hybrid was approximately intermediate

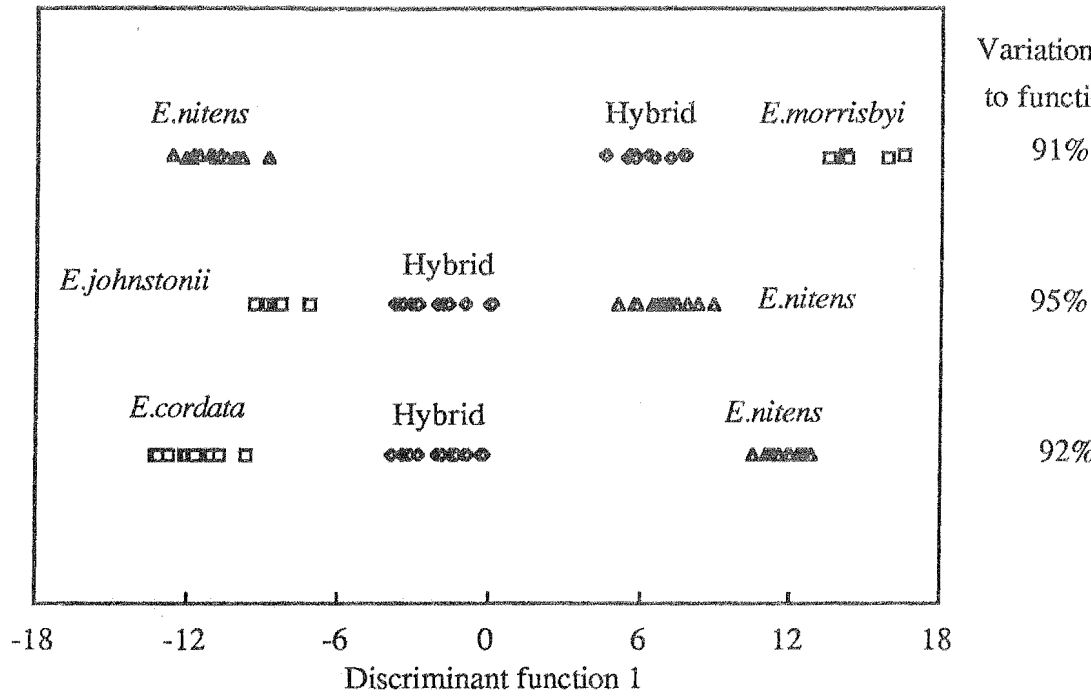


Figure 9.4 Three separate discriminant function analyses using morphological characters from seedlings of *E.nitens* (△), the paternal control (□), either *E.cordata, E.johnstonii or E.morrisbyi,* and hybrids (◇).

Analyses are shown as one-dimensional plots.

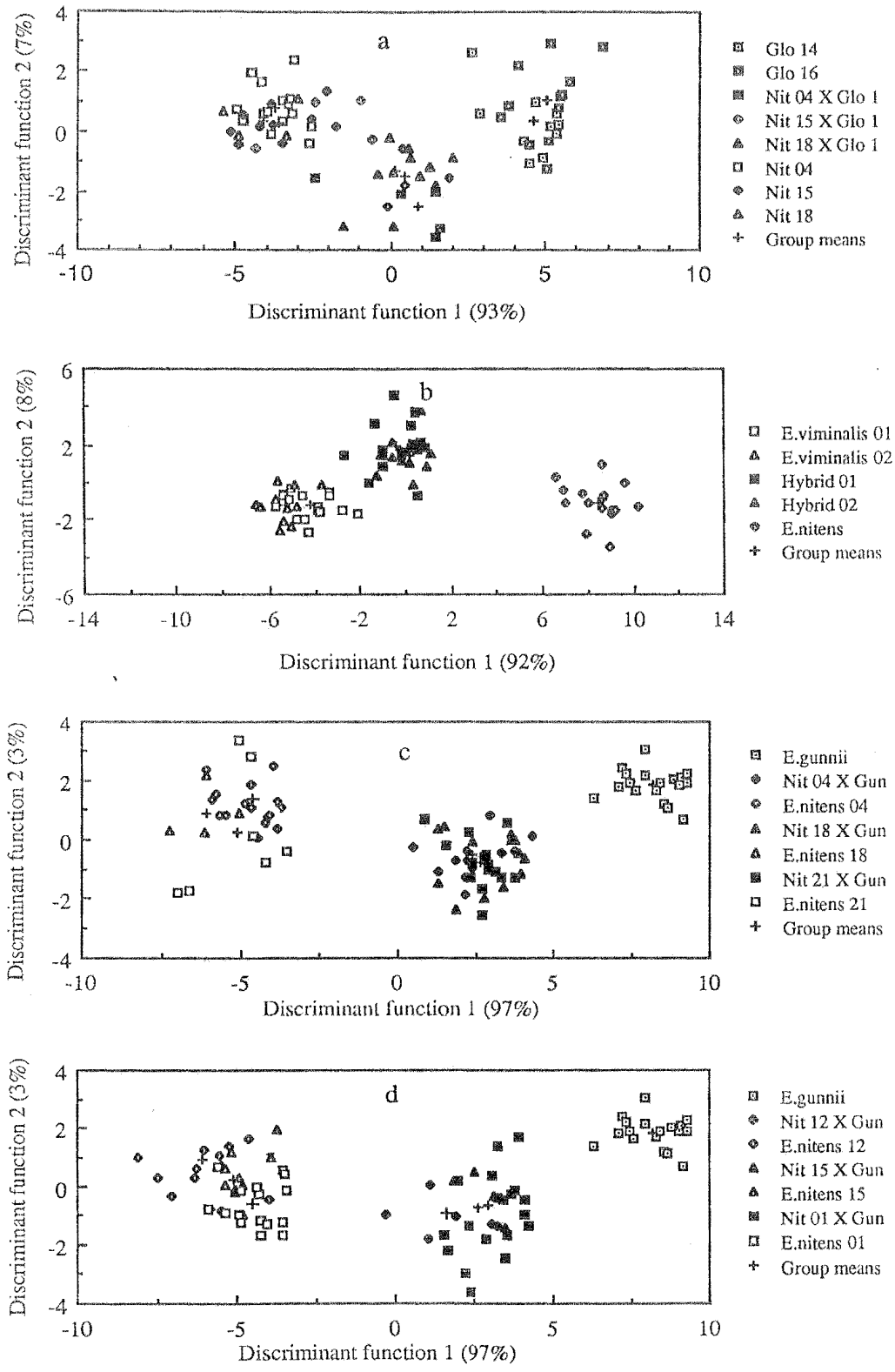


Figure 9.5 Discriminant function analyses using morphological characters from seedlings produced by interspecific controlled crosses, where the maternal parent was *E.nitens* , and the paternal parent was, (a) *E.globulus*, (b) *E.viminalis*, and (c) *and (d) E.gunnii*.

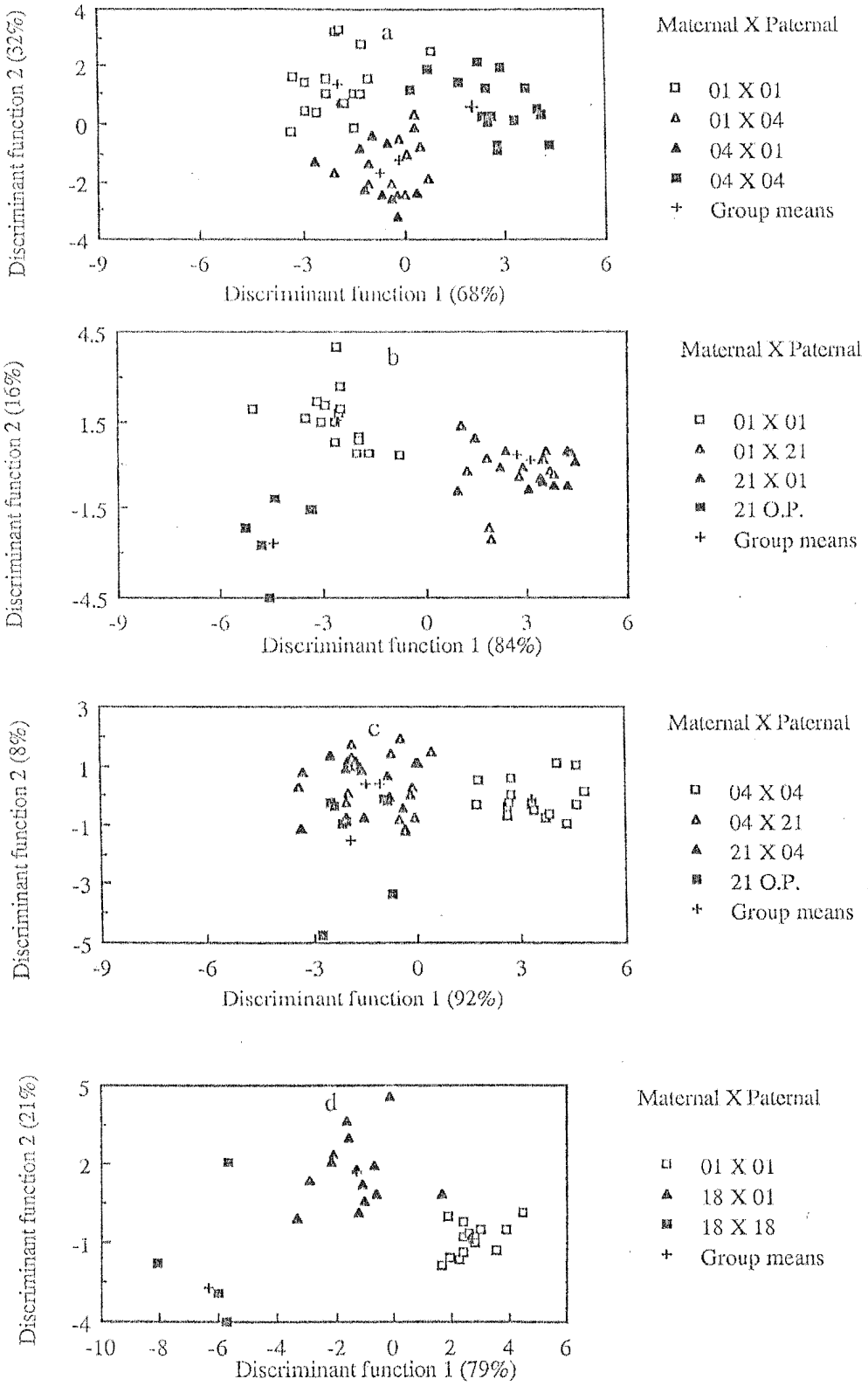


Figure 9.6 Four separate discriminant function analyses using morphological characters from seedlings produced by intraspecific controlled crosses in *E.nitens*.

9.4 Discussion

Viable seed (Table 9.2) has been obtained from both interspecific and intraspecific controlled crosses reported in Chapter 8. This means that breeding programmes with *E.nitens* maternal parents are capable of producing viable crosses involving either widely ranging provenances or other eucalypt species, at least within the series (*Viminales*) as used here. There are therefore the possibilities of combining species that are generally incapable of interbreeding, either through geographic isolation or temporal differences in flowering times. Although most of the interspecific crosses germinated readily, some of them displayed high rates of abnormality (Table 9.2). This may indicate some degree of genetic incompatibility between widely differing species, such as *E.nitens* and *E.gunnii*. However, despite this many seedlings were raised which showed quite reasonable growth, at least whilst in the glasshouse (Table 9.4).

The intermediate nature of the morphology of the hybrids is in agreement with that well accepted in *Eucalyptus* species (Pryor 1976). Leaf and stem glaucousness generally displayed additive inheritance in both interspecific and intraspecific crosses, whether the paternal parent was less glaucous (e.g. *E.johnstonnii*) or more glaucous (*E.gunnii*). There were three slight exceptions, though there was no consistent pattern, in stem glaucousness (i.e. *E.nitens* X *E.cordata*, *E.nitens* X *E.morrisbyi* and Tree 18 X Tree 01). It has been postulated that the genetic control of glaucousness in eucalypts is in many cases relatively simple with controlling genes located at one or more loci (Barber 1955). Additive inheritance has been reported in F_1 hybrids *E.cinera* X *E.robusta* (Pryor 1954) and *E.pulverulenta* X *E.caesia* (Pryor 1956). The findings from this study support those of Pryor. Paton (1981) proposed that data from manipulated crosses between *E.pulverulenta* and *E.grandis* suggest that glaucousness is at least partially recessive. Wiltshire and Reid (1987) when investigating natural hybrids between *E.perriniana* and *E.rodwayi*, reach a similar conclusion. Results from F_1 hybrids in France between *E.gunnii* (glaucous) and *E.ovata* (green) and open-pollinated F_2 led Cauvin *et al.* (1987) to suggest that glaucousness is effectively fully recessive in these species. Stem redness generally displayed a tendency towards dominance and even slight heterosis (particularly Tables 9.3 and 9.4). The relatively high mean scores of some hybrids may be at least due to an underestimate of redness in some parents because of masking effects of stem glaucousness (e.g. *E.gunnii*). Likewise, the relative proportion of laterals (RL) in all crosses appeared to display dominant inheritance (Tables 9.3 and 9.4), except in *E.nitens* X *E.globulus* and intraspecific crosses (Tables 9.4 and 9.5), where there were generally no pronounced differences between parents. The long-term effects of this morphological character may have important bearing on the suitability of some interspecific hybrids in plantation forestry. For instance, if plants with high RL values are also characterised by subsequently poorer branching habits which degrade value, then the overall value of some hybrid combinations may diminish.

Though the long-term growth of the hybrids with respect to parents as yet remains

speculative, if it also proves to be intermediate, then interspecific hybridization may well offer considerable promise in some breeding programmes. One such promising situation is where breeding objectives may be to attempt to combine superior frost resistance (generally found in slower-growing species) and rapid growth (generally found in less frost resistant species). Indeed, interspecific hybrids are already used in some planting programmes (e.g. Pryor 1985; Potts and Potts 1986).

There are now a number of pieces of data indicating a number of mechanisms of inbreeding depression within *E.nitens*. There was some evidence in the previous chapter that percentage of capsules set was lower in self-pollinated flowers than in outcrossed flowers. Results from this chapter suggest that self-fertilised seed may be characterised by lower viability than that of outcrossed seed, and the progeny are inclined to suffer higher mortality (Table 9.2). In addition, the height and sometimes diameter growth of self-fertilised parental controls was generally less than that of outcrosses (Table 9.5). This adds to the growing body of evidence highlighting inbreeding effects within *Eucalyptus* species (Hodgson 1976b,c; Eldridge and Griffin 1983; Potts *et al.* 1987).

Of particular interest is the unusually poor growth of the crosses between Trees 01 and 21. Such abnormally slow growth cannot be attributed to immature seed from Tree 21 (see Chapter 8) since the reciprocal showed similarly abnormal growth. Although these two trees represent provenances with vastly different form, there is no evidence to suggest that this effect is commonplace in crosses involving distinctively different provenances and/or one of these trees. Crosses between Trees 01 and 04, and Trees 04 and 21 (Figure 9.6a,b and Table 9.5) revealed no similar retardation of growth, even though these also involved different provenances (i.e. Tree 01 from Macalister, Tree 21 from Errinundra and Tree 04 from Northern N.S.W.). These abnormal growth effects in an intraspecific cross are particularly surprising in light of the general lack of such abnormality in interspecific crosses (apart from crosses involving *E.gunnii* and *E.johnstonnii*). Obviously a greater number of crosses need to be made within *E.nitens* to see how widespread this phenomenon may be.

Although reasonable separation of intraspecific crosses from parental genotypes was obtained (Figure 9.6), separations were not as clear-cut as in interspecific crosses (Figure 9.4 and 9.5), where the inherent variations between parents were much greater than between *E.nitens* trees. Other characters may need to be used when interpreting differences amongst intraspecific crosses. Of particular relevance to studies within *E.nitens* are biochemical characters such as polyphenolic constituents. Pederick and Lennox (1979) found highly significant differences between forms of *E.nitens* in the patterns of occurrence of these substances. Other biochemical characters may be required for analyses of crosses within a provenance where polyphenolic variation is slight.

CHAPTER 10

FROST RESISTANCE OF CONTROLLED CROSSES

10.1 Introduction

Improvement of frost resistance in *Eucalyptus* species is particularly important in many parts of the Northern Hemisphere (Pryor 1957a; Potts and Potts 1986) where plantings are sometimes exposed to severe winter conditions (Martin 1948; Evans 1983). It may become important in some parts of Australia, e.g. Tasmania, the State with the largest current eucalypt plantation programme (Tibbits 1986). Pryor (1957a) concluded that breeding programmes for frost resistance with eucalypts should follow four lines of research, viz., trials to assess (1) most resistant species, (2) most resistant provenances within species, (3) progeny from natural hybrids, and (4) progeny from manipulated hybrids. The wide variation in frost resistance of various *Eucalyptus* species (the first line of Pryor's programme) and within many species (the second line of Pryor's programme) have been well demonstrated in the present study (Chapters 7 and 6 respectively), particularly when plants are at well developed levels of hardiness. Opportunities for developing improved strains of frost resistant eucalypts, through the use of segregating progeny of natural hybrids or artificial hybridization (lines 3 and 4 of Pryor's programme), have received little attention in Australia. However, as early as 1934, artificial hybridization was used as a means of attempting to obtain frost resistant and productive forms of *Eucalyptus* species in Russia (Pilipenka 1969). Although some valuable data were obtained on the relative frost resistance of the hybrids and their parents, most of this valuable genetic material has been lost due to severe winters. More recently, workers in France have concentrated on interspecific hybridization between fast-growing species with good timber properties (e.g. *E.dalrympleana*) and highly resistant species (e.g. *E.gunnii*) (Potts and Potts 1986). Pryor (1957a) suggested that combinations such as these may produce potentially useful timber trees with relatively high levels of frost resistance.

Despite the large numbers of crosses made in both Russia and France there are little data on the relative differences in frost resistance of hybrids and their parents. Data are particularly lacking on intraspecific crosses. The leaf disc method for assessing relative frost resistance (Chapter 4), and the ability to substantially harden plants under controlled environmental conditions (Chapter 5), means that techniques are available to compare the frost resistance of hybrids and parents, at a number of frost temperatures and at a range of levels of hardiness, with sufficient replication and without risking plant death (as often occurs when frosting whole seedlings). In this Chapter, plants raised from seed produced

by both interspecific and intraspecific controlled pollinations (Chapters 8 and 9), were artificially hardened, and leaf discs frosted to investigate the relative frost resistance of hybrids and parental controls, and the nature of inheritance patterns.

10.2 Materials and methods

Seedlings from interspecific and intraspecific controlled crosses were grown in a heated glasshouse (see Section 9.2) and when 20 cm or more tall were selected for artificial frosting using leaf discs (usually six seedlings from each family). Estimates of relative frost resistance were made in three separate experiments (Table 10.2). The first experiment examined variation amongst a range of interspecific hybrids (six in all, Table 10.3) and their maternal (*E.nitens*) and paternal parents (other *Eucalyptus* species). The second examined the variation amongst six families of the one hybrid type, viz., *E.nitens* X *E.gunnii* (Table 10.4). In the third experiment, the relative frost resistance of 12 intraspecific controlled crosses was assessed (Table 10.5). Parental controls were grown from open-pollinated or self-pollinated seed collected from the parents of the hybrids. Although they are not the true parental genotypes they represent an approximation.

Frost resistance was estimated from the relative leakage of cellular electrolytes from leaf discs, using standard frosting methods (Section 4.2), except that discs of 12 mm diameter were used (because of the much smaller leaves on some of the other *Eucalyptus* species and hybrids, compared with *E.nitens*). Assessments of frost resistance were made after seedlings had been hardening for four and nine weeks in a cabinet at 18°C day/2°C night, 8 h photoperiod (*c.* 300 μmol m^{-2} s^{-1}).

At the commencement of hardening treatments there were large differences in the mean heights of the various controlled crosses (see also Tables 9.3, 9.4 and 9.5). In particular, seedlings from the *E.gunnii* and *E.johnstonii* paternal parents, *E.nitens* (Tree 21) X *E.gunnii* and *E.nitens* X *E.johnstonii* families were much smaller, with fewer expanded leaf pairs, than most of the other families at the commencement of Experiments 1 and 2 (Table 10.1). At the commencement of Experiment 3, other seedlings from the *E.gunnii* paternal parent and the *E.nitens* (Tree 21) X *E.gunnii* family were much taller and were therefore also included in Experiment 3. In this manner the possible effects of vastly different plant size on the development of frost resistance could be further assessed. The results clearly show that the smaller seedlings from these two families (Experiment 2) were significantly ($P<0.05$ and $P<0.001$) less frost resistant than taller seedlings (Experiment 3), whilst at the same time there were no significant differences ($P>0.05$) in the other four families included in more than one experiment (Table 10.1).

Differences between families were analysed at two levels. T50 values were evaluated for each seedling, and family means and least significant differences between family means calculated (presented in the Tables). Levels of significance between two parents and between each parent and their hybrid (e.g. *E.nitens* (maternal), *E.globulus* (paternal) and *E.nitens* X *E.globulus*) were analysed with two-way analysis of variance, using the relative conductivity of the bathing medium at four designated frost temperatures

(arc-sin transformation, Sokal and Rohlf 1981).

Linear regressions and correlation coefficients, between progeny and mid-parent frost resistance, were separately calculated for interspecific crosses, intraspecific crosses and *E.nitens* X *E.gunnii*. Three groups of experimental data were analysed, viz., (1) T50 values after four weeks hardening, (2) T50 values after nine weeks hardening, and (3) T50 values at both durations of hardening.

10.3 Results

10.3.1 Frost resistance of interspecific hybrids

Four weeks after hardening, there were highly significant differences in relative frost resistance amongst the 14 families (interspecific hybrids and parents) in Experiment 1, based on relative leakage of cellular electrolytes or T50 values ($P<0.001$). Families differed by as much as 3.4°C in T50 values (Figures 10.1 and 10.2, *E.gunnii* paternal control included from Experiment 3). Some hybrids were significantly different from one or both parental controls, e.g. combinations involving *E.cordata*, *E.johnstonii* and *E.gunnii* (Table 10.2). Five weeks later, when the overall level of hardiness had increased by 1.8°C, differences in T50 values had increased to -4.9°C (Figures 10.1 and 10.2) and there were highly significant differences between hybrids and parents for all combinations except *E.viminalis* (Table 10.2). The mean frost resistance of hybrids never exceeded that of the more frost resistant parent and was generally intermediate to that of the parents.

Table 10.1 Mean frost resistance and stem height (cm) of seedlings from families of controlled crosses incorporated in more than one of the three hardening experiments. Relative frost resistance evaluated as the temperature resulting in 50% leakage of cellular electrolytes from frosted leaf discs (T50, °C), after four weeks hardening.

Family	Experiment 1		Experiment 2		Experiment 3	
	T50	Height	T50	Height	T50	Height
E.nitens (Tree 01)	-4.8	33.8	-5.6	28.0	-4.9NS†	26.7
E.nitens (Tree 04)	-4.8	28.5	-5.4	28.3	-5.0NS	28.2
E.nitens (Tree 15)	-5.4	35.7	-5.1	27.7	- NS	-
E.nitens (Tree 21)	-	-	-4.9	33.3	-5.3NS	30.0
E.gunnii	-	-	-5.0	28.0	-7.1*	42.7
E.nitens (Tree 21)} X *E.gunnii* }	-	-	-4.1	25.0	-6.8***	37.5

†, significance of differences between Experiments (T50 values within a family); NS, $P>0.05$; *, $P<0.05$ and ***,$P<0.001$.

Table 10.2 Levels of significance from ANOVA (based on relative leakage of cellular electrolytes from leaf discs, at four frost temperatures) comparing relative frost resistance of pairs of families from controlled crosses. Seedlings were artificially hardened for four and nine weeks by growing them at 18°C day (8 h)/2°C night. Levels of significance, NS (P>0.05), * (P<0.05), ** (P<0.01), *** (P<0.001).

Parents	4 weeks hardening			9 weeks hardening		
(maternal, paternal)	M & P†	M & H††	P & H†††	M & P	M & H	P & H
Experiment 1						
E.nitens, E.cordata	**	NS	***	***	NS	**
E.nitens,E.globulus	NS	NS	NS	***	***	NS
E.nitens, E.johnstonii	***	***	NS	***	NS	*
E.nitens, E.morrisbyi	NS	NS	NS	***	***	NS
E.nitens, E.viminalis	NS	NS	NS	NS	NS	NS
E.nitens, E.gunnii	***	*	**	***	***	**
Experiment 2 (all maternal parents *E.nitens*)						
Tree 01,*E.gunnii* A	***	NS	**	***	*	***
Tree 04,*E.gunnii* A	***	NS	NS	***	***	NS
Tree 12,*E.gunnii* A	***	***	NS	***	***	NS
Tree 15,*E.gunnii* A	***	***	*	***	***	**
Tree 18,*E.gunnii* A	*	NS	NS	***	**	NS
Tree 21,*E.gunnii* B	***	***	NS	***	***	*
Experiment 3 (all intraspecific crosses involving *E.nitens*)						
Tree 01, Tree 04	NS	*	**	NS	NS	NS
Tree 01, Tree 05	**	**	NS	**	*	*
Tree 01, Tree 18	***	NS	**	NS	**	***
Tree 01, Tree 21	NS	*	NS	NS	NS	NS
Tree 04, Tree 21	NS	NS	NS	NS	NS	NS

†, comparison between maternal and paternal

††, comparison between maternal and hybrid

†††, comparison between paternal and hybrid

A, data for *E.gunnii* parental seedlings is from Experiment 3

B, data for all three families is from Experiment 3

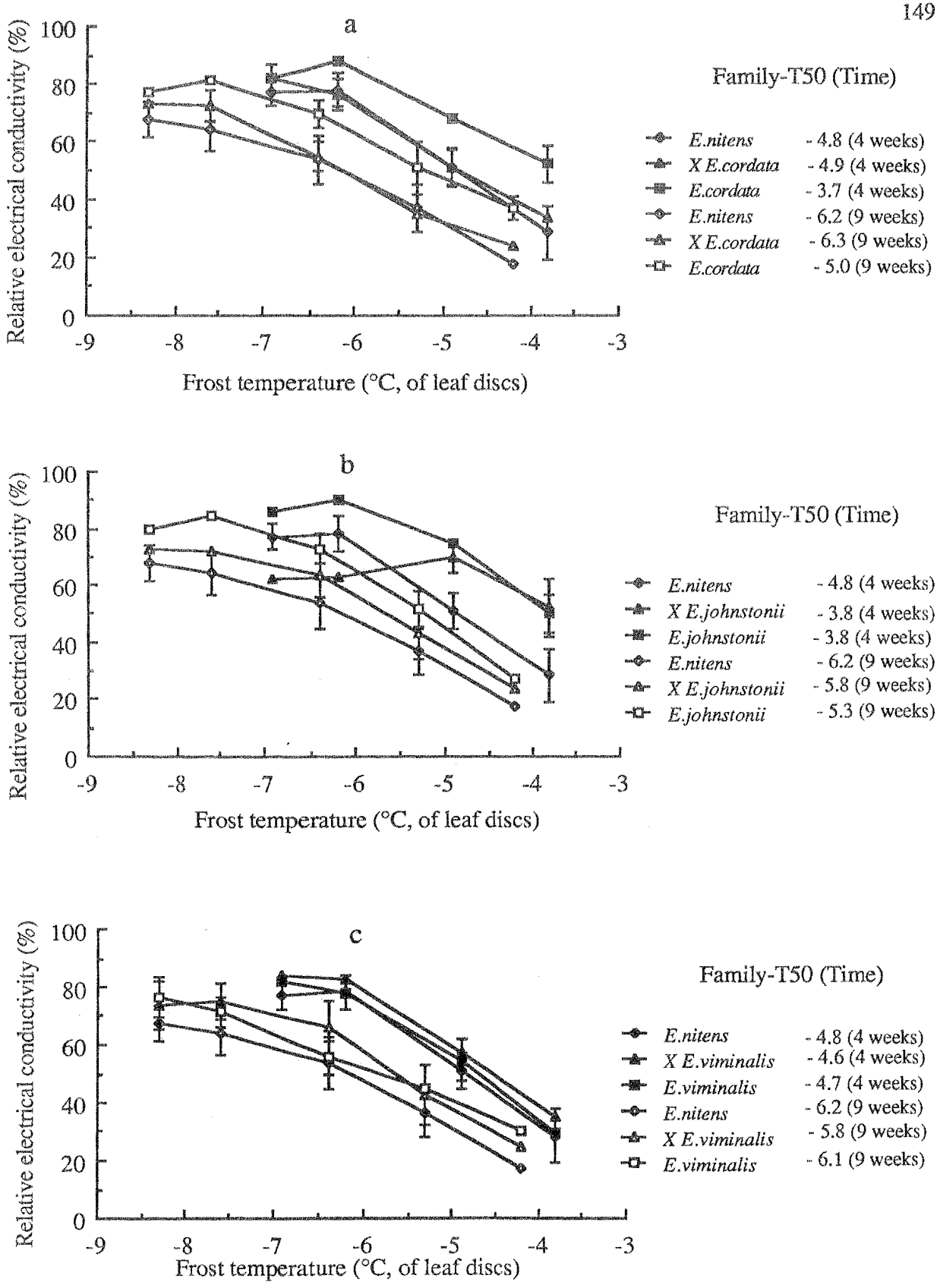


Figure 10.1 Relative frost resistance of interspecific hybrids and parental controls for, (a) *E.nitens X E.cordata*, (b) *E.nitens X E.johnstonii*, and (c) *E.nitens X E.viminalis*.

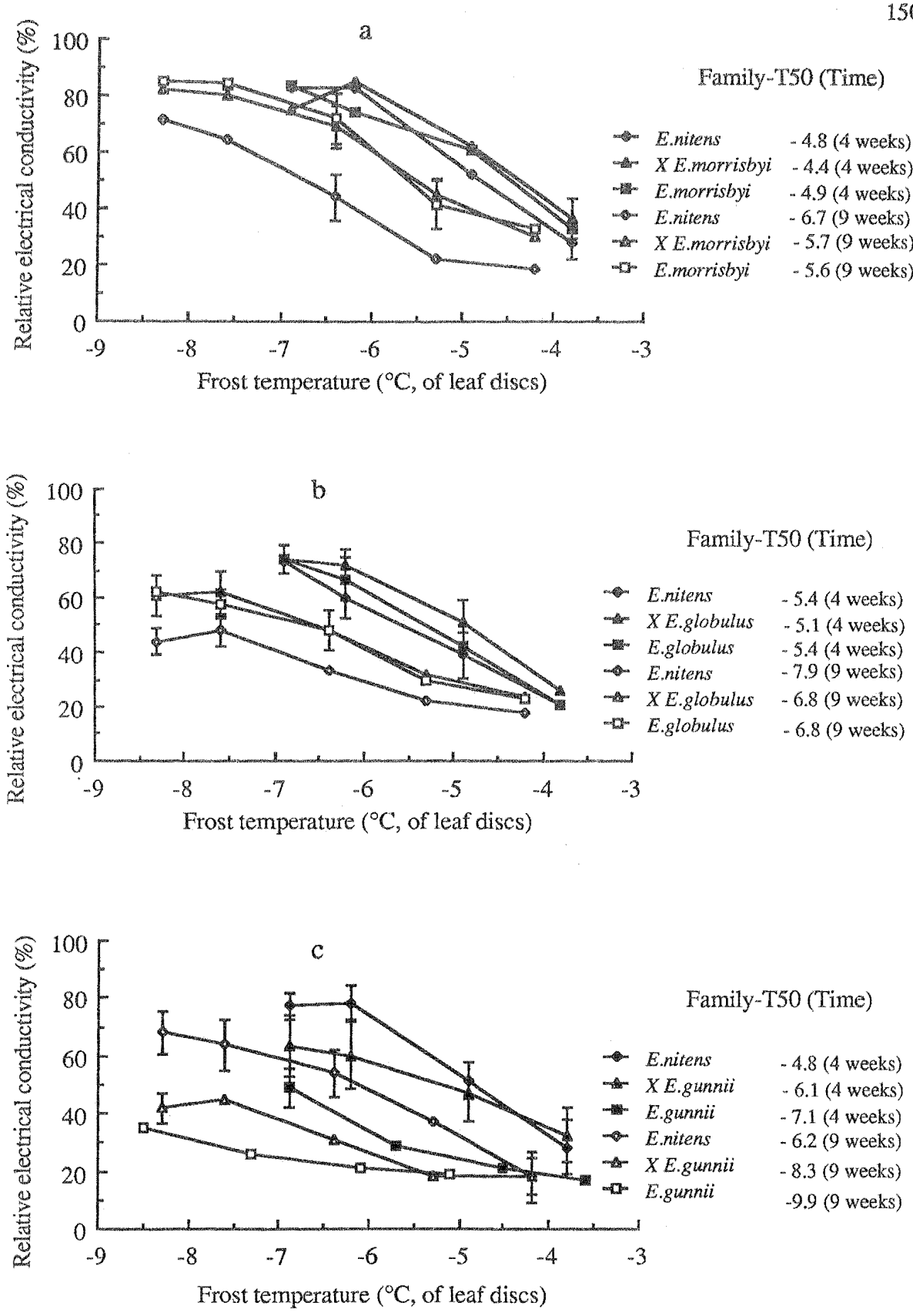


Figure 10.2 Relative frost resistance of interspecific hybrids and parental controls for, (a) *E.nitens* X *E.morrisbyi* (b) *E.nitens X E.globulus*, and (c) *E.nitens X E.gunnii*.

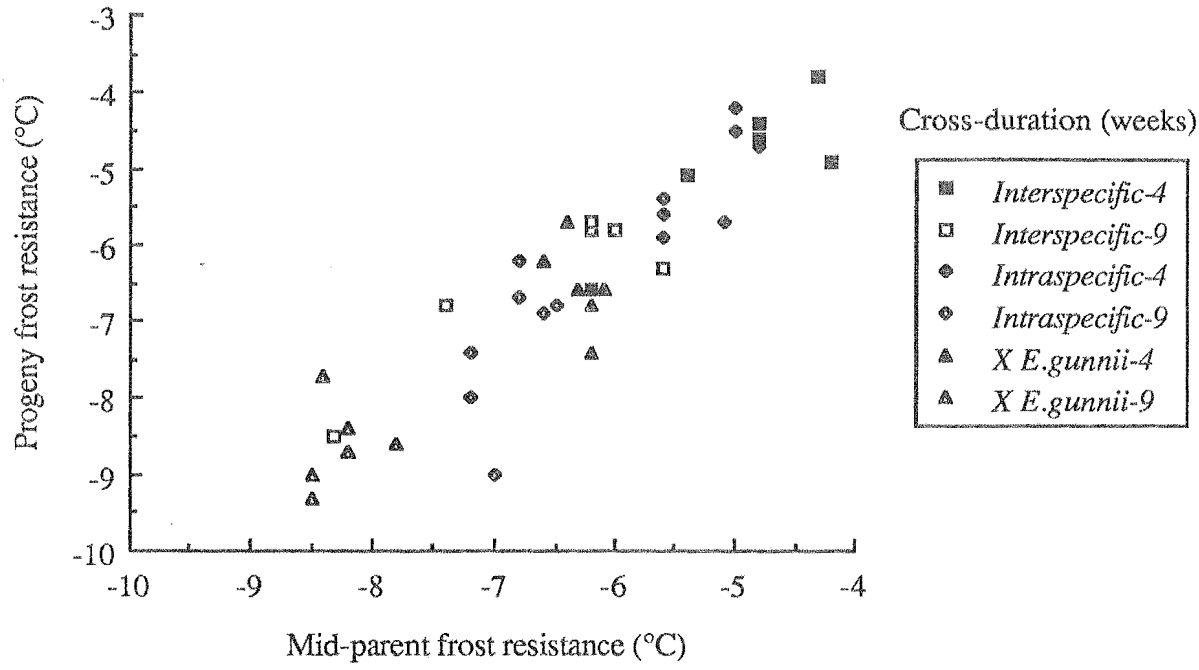


Figure 10.3 Relationship between mean frost resistance of the progeny from controlled crosses and the mean frost resistance of mid-parents.

Table 10.3 Relative frost resistance of various *E.nitens* X *E.gunnii* hybrids (*E.nitens* maternal parent) and their parents. Given are mean temperatures (°C) resulting in 50% leakage of cellular electrolytes from frosted leaf discs of potted seedlings. Seedlings were artificially hardened for four and nine weeks by growing them at 18°C day (8 h)/2°C night.

Maternal	4 weeks hardening			9 weeks hardening		
Tree No	Maternal*	Paternal †	Hybrid	Maternal	Paternal †	Hybrid
NIT 01	-5.6	-7.1	-5.7	-6.9	-9.9	-7.6
NIT 04	-5.4	-7.1	-6.6	-6.5	-9.9	-8.7
NIT 12	-5.3	-7.1	-7.4	-6.1	-9.9	-9.3
NIT 15	-5.1	-7.1	-6.6	-6.4	-9.9	-8.4
NIT 18	-6.2	-7.1	-6.2	-7.1	-9.9	-8.3
NIT 21	-5.3	-7.1	-6.8†	-6.4	-9.9	-8.6†
Mean ± S.D.	-5.3 ± 0.9	-7.1 ±0.7	-6.6 ± 1.0	-6.6 ± 1.2	-9.9 ± 1.0	-8.5± 0.8

*, progeny from self-pollinations, except NIT 21 (progeny from open-pollinations)
†, *E.gunnii* open-pollinated (data from Experiment 3)

The six *E.nitens* X *E.gunnii* hybrids were on average 1.3°C more frost resistant than their *E.nitens* mothers and 0.5°C less frost resistant than their *E.gunnii* father, only four weeks after the imposition of hardening treatments (Table 10.3). After an additional five weeks these differences were 1.9 and 1.4°C respectively, and all hybrids were significantly different from their maternal parents (Table 10.2). In all six families, the frost resistance of the hybrids was intermediate between the parents (Table 10.3).

10.3.2 Frost resistance of intraspecific crosses

There were large differences in the mean frost resistance of the various intraspecific crosses at both durations of hardening ($P<0.001$), with the range being 2.0 and 3.6°C after four and nine weeks hardening respectively (Table 10.4). In a number of cases there were clear differences in the relative frost resistance of some crosses and one of their parents (Table 10.2). There were no significant differences ($P>0.05$) between two of the reciprocal crosses (viz., Tree 01 X Tree 05 and vice versa, and Tree 01 X Tree 04 and vice versa) but Tree 04 X Tree 21 was 1.4°C more frost resistant than Tree 21 X Tree 04 after nine weeks hardening ($P<0.01$, Tables 10.2 and 10.4). Generally speaking, crosses involving Trees 05 or 18 had high levels of frost resistance, and the outcross Tree 18 X Tree 01 showed significantly superior frost resistance to both parents after nine weeks hardening (Tables 10.2 and 10.4).

Table 10.4 Relative frost resistance of various *E.nitens* intraspecific controlled crosses. Given are mean temperatures (°C) resulting in 50% leakage of cellular electrolytes from frosted leaf discs of potted seedlings. Seedlings were artificially hardened for four and nine weeks by growing them at 18°C day (8 h)/2°C night.

Family (*E.nitens* Tree No)		Duration of hardening (weeks)	
Maternal	Paternal	4	9
NIT 01(M)*	NIT 01	-4.9	-6.9
NIT 01	NIT 04	-4.5	-6.2
NIT 01	NIT 05	-5.6	-7.4
NIT 01	NIT 21	-5.7	-6.9
NIT 04(N)*	NIT 01	-4.2	-6.7
NIT 04	NIT 04	-5.0	-6.6
NIT 04	NIT 21	-4.6	-6.8
NIT 05(T)*	NIT 01	-5.6	-8.0
NIT 05	NIT 05	-6.0	-7.6
NIT 18(S)*	NIT 18	-6.2	-7.1
NIT 18	NIT 01	-5.9	-9.0
NIT 21(E)*	NIT 04	-4.7	-5.4
NIT 21	Open pollinated	-5.3	-6.4
Mean ± S.D.		-4.7 ± 0.1	-7.3 ± 1.6
LSD (P<0.01) between families		1.0	1.0

*, provenance of tree, viz., Macalister (M), Northern N.S.W. (N), Toorongo (T), Southern N.S.W. (S) and Errinundra (E).

Table 10.5 Linear regressions performed on frost resistance of progeny (PR) and mid-parent (MP) T50 values. NS, (P>0.05); * (P<0.05) and; *** (P<0.001).

Nº cases	Duration of hardening (weeks)	R^2	Regression	S.E. of slope
Interspecific crosses				
6	4	.743*	PR= 0.50 + 1.09 MP	0.321
6	4	.789*	PR= -0.31 + 0.93 MP	0.241
12	4 and 9	.864***	PR= -0.09 + 0.97 MP	0.121
Intraspecific crosses				
8	4	.636*	PR= 2.60 + 1.48 MP	0.458
8	4	.468NS	PR=10.87+ 2.62 MP	1.144
16	4 and 9	.746***	PR= 1.61 + 1.28 MP	0.200
E.nitens X *E.gunnii*				
6	4	.376NS	PR=-18.78 + 1.94 MP	1.271
6	4	.039NS	PR= -5.26 + 4.06 MP	1.012
12	4 and 9	.748***	PR= -0.35 + 0.99 MP	0.182

10.3.3 Estimates of heritability from regressions

Reasonable correlations (R^2, P<0.05) were obtained between progeny and mid-parent frost resistance for the group of six interspecific crosses (Table 10.5). At both durations of hardening, regression coefficients were about 1.0 (standard errors were about 0.3 in each case) indicating frost resistance is a heritable trait. At either four or nine weeks hardening, poor linear correlations were obtained between progeny and mid-parent frost resistance within, either intraspecific or *E.nitens* X *E.gunnii* cross-types, although the intraspecific correlations were better (Table 10.5). Much better correlations were obtained for all three groups (interspecific, intraspecific and *E.nitens* X *E.gunnii* cross-types), when data from both durations of hardening were used. However, this appears to be largely due to a good relationship between frost resistance at four and nine weeks hardening (Figure 10.3), rather than a good correlation between progeny and mid-parent frost resistance *per se*.

10.4 Discussion

Large differences in relative levels of frost resistance between species, between intraspecific crosses, and between interspecific hybrids and parental controls, have been demonstrated at two levels of hardiness (Figures 10.1 and 10.2 and Tables10.2 to 10.4). Although the findings from the artificial hardening experiments are based on single trees from a number of species, they clearly support earlier findings (Chapter 7) that *E.gunnii* has

a high level of frost resistance. Whilst *E.nitens* is more frost resistant than a number of other species (e.g. *E.cordata*), *E.nitens* itself is inferior to species such as *E.gunnii*.

In this study, previous comparisons of relative frost resistance under controlled environmental conditions used seedlings at comparable stages of development (Chapters 4, 5 and 7). In this chapter, *E.nitens* seedlings from the same family and at comparable stages of development, hardened to approximately equal levels of frost resistance in separate experiments (Table 10.1). However, smaller seedlings of two families (i.e. *E.gunnii* and *E.nitens* X *E.gunnii*) were found to be less frost resistant in a separate experiment than taller seedlings of the same families (Table 10.1). This adds to the body of data highlighting the significance of plant size and/or age and/or health on frost resistance (see Chapters 5 and 7). Such effects are likely to become more critical when comparisons are being made amongst different species and/or hybrids, with vastly different growth rates, rather than amongst provenances and/or families within a species (of somewhat more uniform growth rates). The complicating effects of plant development on frost resistance may explain some of the unexpected variation in other results. For instance, *E.johnstonii* and its hybrid with *E.nitens* were unexpectantly less frost resistant than their *E.nitens* comparison (Figure 10.1b) and they were also quite small compared with many of the other families in Experiment 1 (like the *E.gunnii* seedlings, Table 10.1). Although they were only slightly shorter on average than *E.nitens*, they had much less leaf area (visual observations). If this effect of vigour on frost resistance is also manifest in very tall plants (with large leaf areas) compared with average size plants, it may have resulted in an overestimate in the frost resistance of parental controls such as *E.globulus* and *E.viminalis*, since they were up to 20 cm taller than their *E.nitens* comparisons. These effects need to be more fully addressed. They may be less pronounced in planted seedlings of the same age than in potted seedlings in confined space. For these reasons, it is planned to further examine the relative frost resistance of the hybrids and their parental controls under plantation conditions.

The data clearly show that hybrids can be produced which have superior frost resistance to that of the less resistant parent (Table 10.4, and Figures 10.1 and 10.2) Overall, the inheritance of frost resistance in eucalypts apears to be additive, which supports other workers findings with eucalypts (van Wyke 1976) and other tree species {e.g. tea plants (Tayao 1982)}. In no cases was there evidence of interspecific hybrids being more frost resistant than both parents (Figures 10.1 and 10.2), i.e. heterosis (Wright 1976). Yet, the patterns of frost resistance in the hybrids are variable since in some cases the hybrid was only as resistant as the less resistant parent, e.g. the *E.nitens* X *E.globulus* (Figure 10.2b) and *E.nitens* X *E.morrisbyi* (Figure 10.2a) families were within 0.1°C of their paternal parent, but at least 1.0°C less resistant than their maternal parent after nine weeks hardening ($P<0.001$, Table 10.2). However, the results from the six families of *E.nitens* X *E.gunnii* (Table 10.4) strongly suggests that the frost resistance of the hybrids with respect to their parents are relatively consistent within a hybrid-type. These data generally support the conclusion of Pilipenka (1969) that F_1 hybrids between a frost resistant and a relatively less resistant species have a higher level of resistance than the less resistant parent.

The poor correlations between the frost resistance of progeny and parents, within intraspecific and *E.nitens* X *E.gunnii* crosses (Table 10.5), should not be expected if frost resistance is highly heritable. This may be due to the low number of replications in each regression (at one duration of hardening). Intraspecific crosses were characterised by slightly better regressions. It may be that in crosses between *E.nitens* and *E.gunnii*, the variation in *E.nitens* mothers was hidden in the hybrids because of the superior frost resistance of the single *E.gunnii* parent (3.3°C more resistant at nine weeks hardening). There is a need to examine these aspects of a larger number of samples, involving more maternal and paternal parents.

There are two main differences between the data of Pilipenka (1969) and that collected in this study. Firstly, Pilipenka (1969) assessed frost resistance following natural frosts (-7.5 to -9.0°C) over three seasons, whilst these studies used artificial frostings with precise control. Secondly, the species reported are generally less resistant than some of those used here, e.g. *E.viminalis* and *E.camaldulensis* c.f. *E.gunnii* and *E.nitens*. Nonetheless, the data from both studies suggest that similar patterns of frost resistance are expressed in hybrids between species differing in frost resistance, whether the most resistant parent is very resistant (e.g. *E.gunnii*) or only moderately resistant (*E.viminalis*). Whilst information on growth of interspecific crosses is scant, it may be that relative growth is also generally intermediate, with respect to both parents, as generally occurs in many morphological (Chapter 9) and some physiological characters (this Chapter). Therefore, it appears that crossing fast-growing species (generally having desirable timber qualities) with more frost resistant species (often with less desirable growth and timber quality) as proposed by Pryor (1957a) and currently practiced in France (Potts and Potts 1986), may well produce genotypes with overall good growth and frost resistance.

The frost resistance of intraspecific crosses (Table 10.4) display similar patterns to those of interspecific crosses (Figures 10.1 and 10.2 and Table 10.3). Intraspecific crosses involving a relatively resistant parent were more resistant than the less resistant parent, e.g. combinations of Trees 05 and 01. Two interesting points arose at nine weeks hardening. Firstly, the apparent heterosis in Tree 18 X Tree 01, and secondly the significantly lower frost resistance of Tree 21 X Tree 04 compared with Tree 04 X Tree 21. The superior frost resistance of Tree 18 X Tree 01 (Table 10.4) is approximately equal to the mean frost resistance of the *E.nitens* X *E.gunnii* hybrids (Table 10.3). This suggests that there may be scope to significantly improve frost resistance within a species without turning to interspecific crossing. Such an approach may have the additional benefits of maintaining relatively higher growth rates than in interspecific crosses, where one parent may be slow growing (e.g. *E.gunnii*).

The seasonal frost resistance of intraspecific and interspecific crosses will be further assessed (and early growth rates will also be measured) in trial plantings established with progeny from these crosses. This additional information will help understand inheritance of frost resistance in eucalypts more fully. Such an approach offers the opportunity to make selections of desirable genotypes, based on field performance (e.g. frost resistance and productivity). Selected clones may be vegetatively propagated if possible.

CHAPTER 11

MICROSCOPIC ASPECTS OF FROST RESISTANCE

11.1 Introduction

Although there is much information on frost damage to *Eucalyptus* species, the manifestations of frost injury at the cellular level and the molecular basis of seasonal changes in resistance remain largely unresearched. Freezing injury appears to be closely linked with membrane disruption and damage (Lyons et al. 1979). Indeed, the use of the conductivity method to successfully assess relative frost resistance in many plant species, including eucalypts, is based on the assumption that freezing injury results in injury to membrane transport properties (Palta et al. 1977a,b) or rupture (Palta *et al.* 1982).

However, the nature of membrane changes associated with freezing injury have been investigated in only a few species, such as the crop species, *Solanum* spp. (potato). Li and Palta (1978) observed the results of frost injury in parenchyma cells of *Solanum* species using electron microscopy and noted that such cells appeared to be quite different to unfrozen controls. In particular, freeze injured cells were missing a large central vacuole and the plasmalemma was separated from the cell wall. However, Palta and Li (1978) found that organelle membranes were not necessarily disrupted at temperatures close to killing temperatures, although protoplasm was swollen. It was only with rupture of the tonoplast and/or plasmalemma that the structural integrity of chloroplasts and mitochondria were affected. They proposed an "hypothesis for a possible sequence of events leading to cell death" in which the active transport system was viewed as the primary site of freezing injury. More recently, Palta et al. (1982) found that relatively large ionic leakages resulting from a frost can occur with little change in cellular ultrastructure. For instance, rupture of cellular membranes was not noticed in *Allium cepa* L. and *S.tuberosum* until 69 and 80% leakage of cellular electrolytes, respectively.

Of the numerous postulated mechanisms causing increased frost resistance in plant species (see Levitt 1980) anatomical and particularly ultrastructural characteristics have been frequently investigated, in order to identify both hardy species and factors correlated with increases in frost resistance. Li and Palta (1978) examined a number of *Solanum* species and found that in general the most frost resistant species had more layers of palisade cells, smaller mesophyll cells and greater stomatal density than less resistant species.

A number of workers have reported cytological changes in plant cells associated with changing levels of frost resistance. Pomeroy and Siminovitch (1971) working with phloem parenchyma cells of black locust (*Robinia pseudoacacia* L.) and Niki (1982) working

with cortical parenchyma cells of Mulberry (*Morus bombyciz* L.), (both deciduous species capable of resisting <-70°C in winter) reported sequences of ultrastructural changes associated with changing levels of frost resistance. In particular, increasing levels of frost resistance were characterised by alterations in the plasmalemma either through invaginations of the membrane (Pomeroy and Siminovitch 1971) or fusion of microvesicle with the plasmalemma (Niki 1982). Using antimetabolites Niki and Sakai (1983) found that the sequence of ultrastructural changes was necessary to acquire freezing resistance in mulberry. In addition, cytoplasmic strands were found to extend across the vacuole with increased frost resistance, and subsequently diminish with decreasing frost resistance (Pomeroy and Siminovitch 1971). Conspicuous seasonal changes in leaf cell ultrastructure have also been observed in spruce {*Picea abies* (L.) Karst. (Senser *et al.* 1975)} and potato (Chen *et al.* 1977b). Starch grains disappeared and the number of osmiophilic globuli increased in chloroplasts as frost resistance increased in *S.acaule* (Chen *et al.* 1977b). Similar trends were observed in spruce (Senser *et al.* 1975). Starch levels were also found to decrease in black locust with increased winter hardiness (Pomeroy and Siminovitch 1971).

The objectives of these investigations were to observe the nature of alterations to cellular membranes in eucalypts with both, frost damage and frost hardening.

11.2 Materials and Methods

Leaf tissue was sampled from fully expanded, healthy juvenile foliage from each of the three species *E.nitens*, *E.perriniana* and *E.gunnii* for preparation for transmission electron microscopy (TEM) investigations. For each species tissue was sampled from an unhardened seedling (growing under outdoor Hobart conditions in early April) and one artificially hardened seedling (two months of 20°C (8 h) day/2°C night; lighting *c.* 300 μmol m^{-2} s^{-1}). One disc (14.5 mm diameter) was cut from each seedling and kept at room temperature, in a vial with 8 ml distilled water, whilst a second disc was cut from the unhardened seedlings and frosted to -3.7°C, under standard procedures (see Section 4.2).

Two hours after the conclusion of frosting, samples were taken from all nine discs for preparation for TEM studies as follows. Pieces, *c.* 1 mm^2, were cut and fixed in 3.5% gluteraldehyde (in 0.1 M phosphate buffer, pH 6.9) at 4°C for 15 h, after which they were rinsed in the phosphate buffer for 1 h before being post-fixed in 2% OsO_4 for 2 h. The samples were again rinsed in the buffer and then dehydrated at 4°C in an acetone series (25%, 50%, 75%, 95% and 100%). Dehydration times were 2 x 30 min at each concentration to 75%, at which they were stored overnight, and thereafter 2 x 30 min at 95 and 100%. Samples were then immersed in 50% low-viscosity epoxy (Spurr 1969)/50% acetone for 2 h, followed by 67% low-viscosity epoxy/33% acetone for 2 h, and finally 100% low-viscosity epoxy. The low-viscosity epoxy was replaced with a fresh mixture after 24 h and after a further 48 h, and the samples then embedded in the fresh epoxy by heating to 70°C for 18 h.

"Thick" sections (*c.* 1 μm thickness) were cut (from trimmed blocks) on a ultratome

with glass knives, and mounted on slides for light microscope observations. These "thick" sections were stained with aqueous toluidine blue (30 sec). "Thin" sections (*c.* 0.1 μm thickness) were cut on the ultratome with a diamond knife, placed on 300 mesh grids and double stained with 2% aqueous uranylacetate (90 min) and 1% lead citrate (10 min). Observations were made using a Hitachi H300 electron microscope and photographic records taken.

To compare frosted and unfrosted tissues, mesophyll cells were classified into three types according to the arrangement of chloroplasts and protoplasm (see Figure 11.1). For each species, the number of mesophyll cells in each category were counted in each mesophyll layer of three to five "thick" sections, of each treatment. Frosted and unfrosted treatments were compared using the G statistic (Sokal and Rohlf 1981). To compare chloroplasts, about 20 chloroplasts were randomly selected and their overall size, the number and size of starch grains and the number of osmiophilic globuli assessed.

11.3 Results

11.3.1 Light microscope observations

Generally speaking, cellular organisation was similar in *E.nitens*, *E.perriniana* and *E.gunnii* , e.g. five or six layers of mesophyll cells and large air spaces. The three species behaved quite differently to the artificial frost, yet there were generally consistent differences between hardened and unhardened tissue irrespective of species.

The -3.7°C frost produced 62% leakage of cellular electrolytes from *E.nitens* leaf tissue, whilst leakage from *E.perriniana* and *E.gunnii* were 34 and 43% respectively (Table 11.1). The relative superior frost resistance of *E.perriniana* and *E.gunnii* over *E.nitens* in an unhardened condition agrees well with that found in trial plantings (see Chapter 7). Unfrosted controls always had <25% leakage of cellular electrolytes.

The previously established correlation between relative electrolyte leakage and leaf damage established for *E.nitens* (Figure 4.6) would suggest that *E.nitens* was more likely to have suffered severe damage than cells of the other two species. Mesophyll cells of unfrosted (unhardened) controls were typically characterized by a thin layer of heavily stained protoplasm containing many chloroplasts, around what appeared to be a large, clear, central vacuole (Figure 11.2). This was confirmed by observations at much higher magnification with the TEM (see below). However, in frosted tissue the heavily stained protoplasm of mesophyll cells appeared detached from the cell wall and somewhat coagulated (Figure 11.1). This appearance is commonly known as "frost plasmolysis" (see Li and Palta 1978). Of over 700 cells assessed in both unfrosted and frosted *E.nitens* tissue, on average 13% and 81% respectively displayed "frost plasmolysis" (Table 11.1). These differences were highly significant ($G=826$, $P<0.005$). In contrast, frosted tissue of *E.perriniana* and *E.gunnii* was characterised by approximately the same proportion of cells with frost plasmolysed appearance as unfrosted tissue (Table 11.1). This suggests that leakage of cellular electrolytes from frosted tissue is not necessarily due to "frost plasmolysis" (assumed

membrane rupture).

In contrast to the relatively clear vacuole in unhardened (unfrosted) mesophyll cells, a large proportion of hardened mesophyll cells was characterised by an intensely stained vacuole ($P<0.01$), in addition to the protoplast (Figure 11.3, Table 11.1). All three species showed similar trends. There were no clear trends in the proportion of epidermal cells which stained heavily (Table 11.1). Generally, the lower epidermis (higher stomatal frequency) had a higher percentage of cells which stained intensely.

Table 11.1 Summary of observations with light microscope and estimates of relative frost resistance for leaf tissue samples from *E.nitens* ,*E.perriniana* and *E.gunnii* seedlings. Unhardened seedlings were growing in a glasshouse (*c.* 25/15°C) and hardened seedlings had been grown for two months at 20°C(8 h) day/2°C night.

Species/treatment	Relative conductivity (%)	T50 (°C)	Percentage cells showing Plasmolysis	Heavily stained vacuole	
				Epidermal	Mesophyll
E.nitens					
Unhardened/frosted	62	>-3.7	81	-	-
Unhardened/unfrosted	24	>-3.7	13	60	9
Hardened/unfrosted	18	-7.3	-	88	92
E.perriniana					
Unhardened/frosted	34	< -3.7	5	-	-
Unhardened/unfrosted	23	< -3.7	2	4	3
Hardened/unfrosted	14	<-10.0	1	50	80
E.gunnii					
Unhardened/frosted	43	*c.* -3.7	13	-	-
Unhardened/unfrosted	16	*c.* -3.7	1	75	2
Hardened/unfrosted	22	-8.7	1	66	55

Table 11.2 Summary of ultrastructural observations with transmission electron microscope for leaf tissue samples from unfrosted *E.nitens*, *E.perriniana* and *E.gunnii* seedlings. Unhardened seedlings were growing in a glasshouse (*c.* 25/15°C) and hardened seedlings had been grown for two months at 20°C(8 h) day/2°C night.

Feature	Unhardened			Hardened		
	E.nitens	*E.perriniana*	*E.gunnii*	*E.nitens*	*E.perriniana*	*E.gunnii*
Starch grains						
per chloroplast	2.2	0.9	1.7	0.5	0.7	1.1
mean length (μm)	16.6	6.0	7.4	9.6	4.4	6.6
Chloroplast						
mean length (μm)	8.9	11.7	8.8	7.5	7.4	7.8
Osmiophilic globuli						
per chloroplast	6.7	10.8	15.1	6.1	18.6	11.2

11.3.2 Transmission electron microscope observations

Frosted *E.nitens* mesophyll cells clearly showed alteration in cellular ultrastructure (Figure 11.4). The plasmalemma was separated from the cell wall and the tonoplast had been also ruptured. TEM observations confirmed the light microscope observations that the ultrastructure of leaf mesophyll cells was characterised by a large central vacuole and a thin, surrounding layer of protoplasm which contained many chloroplasts. Resolution of more detailed cellular ultrastructure was often possible. This was particularly so for the chloroplasts where starch grains, granum and osmiophilic globuli were usually conspicuous (Figure 11.5). Details of cell walls and plasmalemma were generally obvious. Tonoplast, nuclei and mitochondria were sometimes seen.

There was one major difference between unhardened and hardened mesophyll cells of the three species. Although starch grains had not fully disappeared from chloroplasts of hardened cells they were noticeably thinner and shorter and generally less frequent than those in unhardened cells (Table 11.2). Many unhardened chloroplasts had a small thylakoid system and a corresponding large reserve of starch (see Figure 11.4). In addition, chloroplasts were slightly shorter in hardened cells, possibly due to a reduction in starch grain content. However, there were no consistent differences between hardening treatments in number of osmiophilic globuli per chloroplast. Indeed, the number of osmiophilic globuli per chloroplast was highly variable varying from four to over 20 in each treatment. Transmission electron micrographs revealed that the heavily stained vacuole appeared as a granular matrix (Figure11.5). However, resolution was not fine enough to enable identification of cytoplasmic strands in the vacuole, should they exist.

11.3 Discussion

In *Eucalyptus* species, cell membranes are clearly a main target of frost injury (Table 11.1, Figure 11.5). This adds to the growing body of data attributing membrane damage as a primary cause of freezing injury to plant species, e.g. Li and Palta (1978), Palta and Li (1978), Rajasheka *et al.* (1979), Steponkus and Wiest (1979), Palta *et al.* (1982).

Severe damage is characterised by major lesions to the tonoplast and plasmalemma (Figure 11.5). However, observations suggest that there may also be other sites and manifestations of injury to cellular membranes associated with less severe damage. For example, electrolyte leakage from frosted *E.perriniana* and *E.gunnii* was not due to membrane rupture. This supports the conclusion of Palta *et al.* (1982) that the nature of incipient injury after a freeze-thaw cycle, as measured by ion efflux, is not necessarily due to disruption of membrane semipermeability or membrane rupture. Indeed, relatively large percentages of electrolytes leaked out of onion and potato cells following a freeze-thaw cycle without any alteration in cellular ultrastructure (Palta *et al.* 1982). In this study, up to 43% of cellular electrolytes leaked out of *Eucalyptus* tissue with no clear membrane lesions.

It is therefore important to re-evaluate the use of the electrical conductivity method for determining frost resistance in *Eucalyptus* species. The results demonstrate that leakage of electrolytes from basically unhardened leaf tissue is largely due to membrane rupture for relative conductivities above 50% (Table 11.1). Such membrane damage should invariably lead to cell death. However, lower relative leakage of electrolytes may be due to other sites of injury, such as disruption to active transport systems (Palta *et al.* 1977a) which may or may not lead to death depending on the extents of injury and/or recovery (see Palta *et al.* 1977b). In this study, the use of the frost temperature causing 50% leakage of cellular electrolytes seems an acceptable index of injury for evaluation of relative frost resistance, based on this TEM evidence and the relationships shown in Chapters 4, 5 and 7 between relative leakage of cellular electrolytes and damage to whole plants.

The present study reveals changes in cellular ultrastucture between unhardened plants (growing outside) and hardened plants (growing under 20°C day/2°C night, 8 h photoperiod of 300 μmol m^{-2} s^{-1}) (Table 11.2). It is not known if these differences were due to low temperature hardening and/or the effects of different light conditions of the two treatments. Relative amounts of starch grains in chloroplasts were reduced in all three species, with artificial hardening treatments. Similarly, starch grains were found to disappear in the chloroplasts of artificially hardened *S.acaule* (Chen *et al.* 1977b). Likewise, reductions in starch grains have been reported following natural hardening in phloem cells (Pomeroy and Siminovitch 1971) and leaf chloroplasts (Senser *et al.* 1975). The disappearance of starch grains may afford a cyroprotective effect if they are converted to soluble sugars, since Garber and Steponkus (1976) found that sucrose provided some protection to thylakoids from frost damage.

Senser *et al.* (1975) and Chen *et al.* (1977b) reported increases in number of osmiophilic globuli in chloroplasts associated with increases in frost resistance. However, in

the present study the number of osmiophilic globuli per chloroplast were found to be extremely variable, even within a single cell, and the only clear trends were lower frequencies in *E.nitens*. However, it is unlikely that the number of osmiophilic globuli per chloroplast *per se* have significant bearing on frost resistance since hardened *E.nitens* had less than half the number that unhardened *E.perriniana* and *E.gunnii* had.

The heavier staining of the vacuole in hardened cells may be related to extension of the cytoplasm. Siminovitch and Levitt (1941) suggest that extended protoplasm may reduce contraction-induced dehydration stresses associated with the freeze-thaw cycle. In addition, increases in numbers of cellular organelles and alterations in plasmalemma have been observed by a number of workers with increases in frost hardiness (Pomeroy and Siminovitch 1971; Chabot and Chabot 1975; Niki 1982; Niki and Sakai 1983). More detailed research into these aspects in eucalypts is necessary. In particular, plants should be grown under controlled environmental conditions where the effects of low temperature and light conditions can be examined separately.

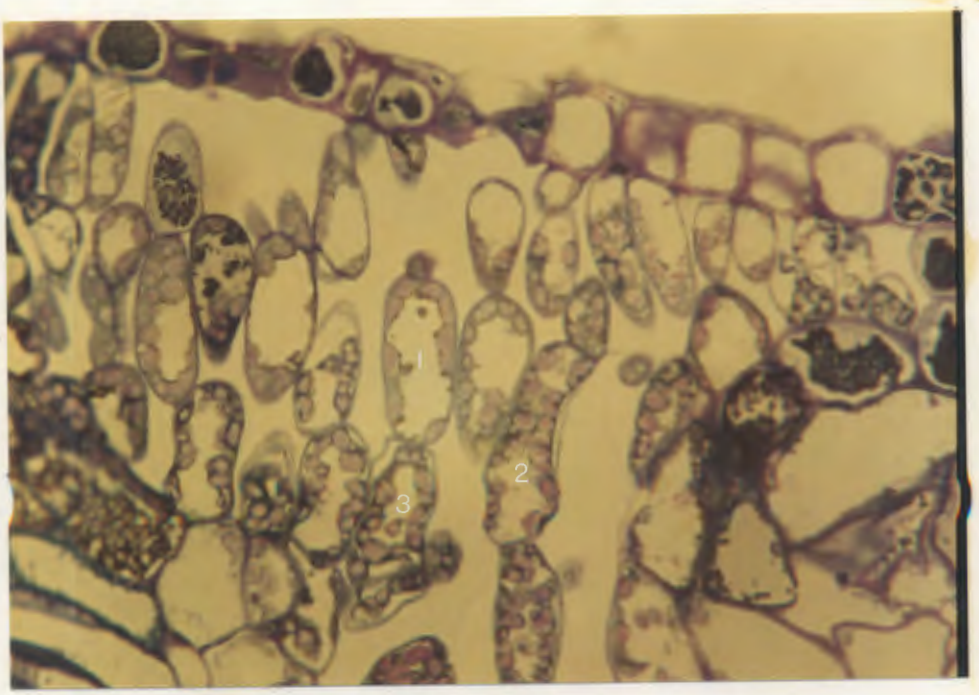


Figure 11.1 Light microscope photograph (X 625) of cross-section of frosted, unhardened *E.nitens* leaf tissue. Mesophyll cells typical of the arrangement of chloroplasts are indicated, viz., (1) chloroplasts appressed to cell wall, (2) chloroplasts slightly separated from cell wall, and (3) all chloroplasts separated from cell wall.

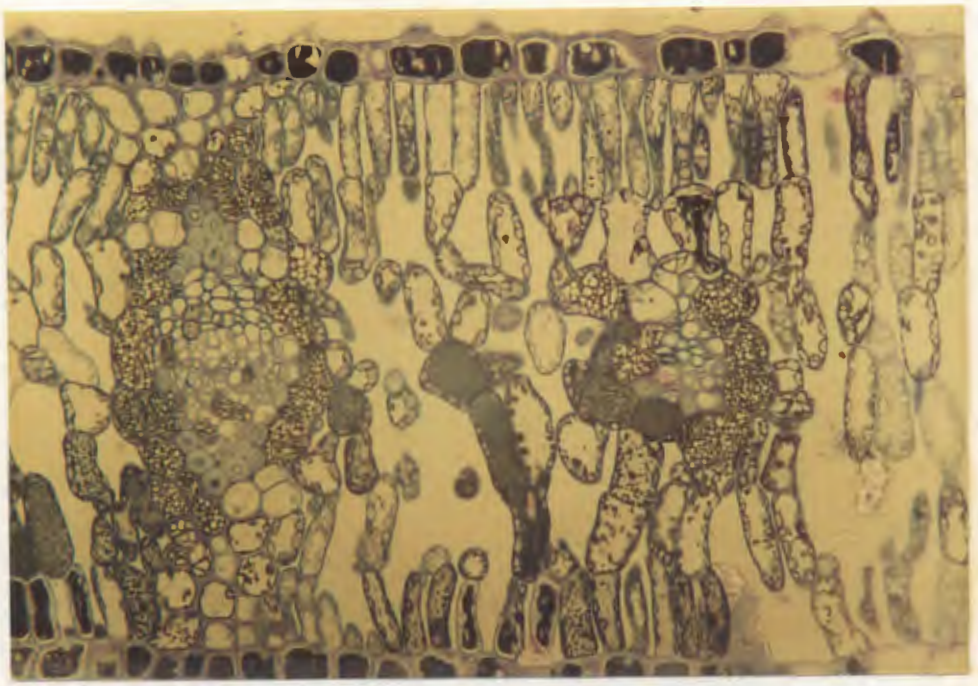


Figure 11.2 Light microscope photograph (X 313) of cross-section of unfrosted, unhardened *E.nitens* leaf tissue.

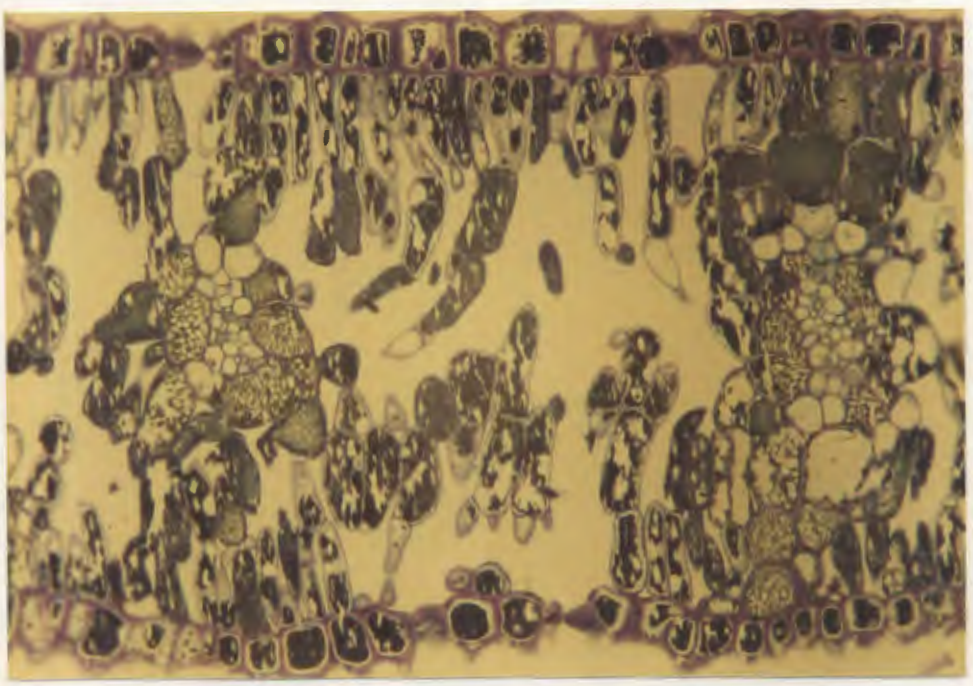


Figure 11.3 Light microscope photograph (X 313) of cross-section of unfrosted, hardened *E.nitens* leaf tissue.

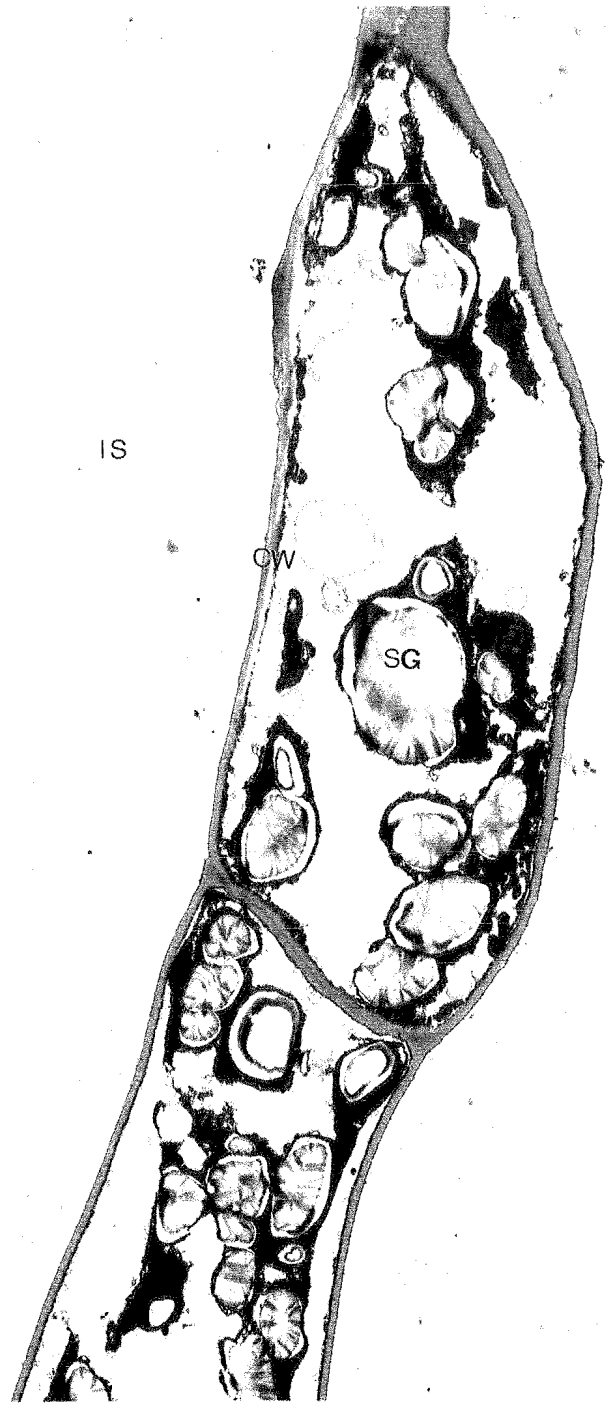


Figure 11.4 Transmission electron micrograph (X 3200) of an *E.nitens* leaf mesophyll cell, from a frosted disc, from an unhardened seedling. IS= intercellular space, CW= cell wall, SG= starch grain.

Figure 11.5 Transmission electron micrograph (X 30 000) of the cell wall and a chloroplast, in a leaf mesophyll cell of an *E.nitens* seedling, grown under artificial hardening conditions (see Table 11.2). IS= intercellular space, CW= cell wall, PL= plasmalemma, G= granum, OG= osmiophilic globuli, SG = starch grain, T= tonoplast, V=vacuole.

CHAPTER 12
DISCUSSION AND CONCLUSIONS

12.1 Discussion

In this study, results from both artificial frosting studies and field trials indicate that frost is a potentially damaging factor to a number of *Eucalyptus* species. Overseas experience is that all eucalypts eventually succumb to the effects of frost, given low enough temperatures (Turnbull 1981; Evans 1983). Indeed, on certain sites subjected to specific environmental conditions, frosts of given severity may be so critical that the use of various species are precluded {e.g. *E.fraxinoides* at Racecourse Plains trial (Table 7.1) or *E.regnans* in the British Isles (Martin 1948)}. Determination of relative levels of frost resistance in *Eucalyptus* seedlings have been made by assessment of damage following both natural and artificial frosts. Whilst the natural frosts are quite capable of differentiating species (Table 7.1) and provenances and/or families (Table 6.4), the overall suitability of this approach is restricted for a number of reasons. Firstly, it relies on the occurrence of discriminating frosts (for example, this did not occur at the Hampshire field trial). Secondly, results do not always indicate the extent of differences between the plants tested, because damage at a range of frost temperatures is required. Thirdly, the risk of loss of valuable genotypes is high, and fourthly, results are not necessarily obtained quickly. In contrast, artificial frosting studies, and particularly those using tissue samples, are not only capable of differentiating species (Table 7.3, Figure 10.1, Table 10.2), provenances (Table 6.4a) and families (Tables 6.6, 10.3 and 10.4), but are largely free of many of the restrictions inherent in waiting for natural frost damage to field plantings. For instance, the extent of differences between plants can be quantified since frosts of known conditions can be used, and the results are obtained reasonably quickly without threatening plant survival. For example, it took 10 months from sowing seed until data on natural frost damage could be collected from the field trials reported in Chapters 6 and 7. Assessment of the relative frost resistance of the same 121 seedlots using leaf discs would only have taken six months at a conservative estimate (12 plants per seedlot artifically hardened for two months and then tested). This is not to suggest that field trials are not required, since there may be other important aspects of plant growth and development that are best assessed in field trials, e.g. tree form and volume growth. Information on seasonal patterns in frost resistance and interactions with environmental factors can be obtained from field trials. In addition, field trials provide a reliable check of laboratory assessments.

The method of artificially frosting leaf discs from eucalypt leaves and assessing

relative frost resistance based on the relative leakage of cellular electrolytes used in this study, has consistently shown good correlation with expression of damage in whole plants. This was demonstrated by two separate methods. Firstly, potted seedlings were artificially frosted and subsequently assessed for leaf damage and relative leakage of cellular electrolytes (Figure 4.7). Secondly, comparisons were made of relative frost resistance of plantation trees by artificially frosting leaf discs and assessing natural frost damage (Figure 7.4a). Furthermore, these correlations appear valid at a range of levels of hardiness, since the potted seedlings with which the correlations were established were unhardened and the planted trees were naturally hardened. The suitability of this method for use with *E.nitens* and other *Eucalyptus* species is in agreement with that found for *E.delegatensis* (Hallam 1986; Raymond *et al.* 1986) and *E.regnans* (Raymond *et al.* 1986). It proved most desirable for determining relative frost resistance of the progeny from controlled crosses (Chapter 10).

Transmission electron microscope studies suggest that cellular membranes are severely damaged at frost temperatures causing more than 50% leakage of cellular electrolytes (Table 11.1). This is further evidence supporting the validity of the leaf disc technique for evaluating frost resistance in eucalypts. Further TEM research should be directed towards observing the nature of alterations to cellular membranes at a range of levels of frost damage, e.g.what are the effects on mitochondria and chloroplast membranes? In addition, ultrastructural changes associated with frost hardening and dehardening require much more detailed investigations. Once ultrastructural changes have been identified, their overall relationship to frost resistance could be examined by artificially interfering with the normal hardening processes by using anti-metabolites (e.g. see Niki and Sakai 1983).

Levels of frost resistance in *Eucalyptus* species are clearly dependent upon both environmental factors and the inherent genetic capacity of individual plants, both of which interact to bring about specific levels of resistance. To a lesser extent, frost resistance varies with other factors such as tissue type (Tables 5.5 and 6.3) and plant size and ontogeny (Table 7.5).

Of the environmental factors affecting levels of frost resistance in eucalypts, the growth treatment, and particularly the temperature regime to which plants are exposed before frosting, assume major importance. Exposure to different temperature regimes can result in either an increase (hardening) or decrease (dehardening) in level of hardiness. Overall, results indicate that a decrease or increase in temperatures (relative to that of other plants or that previously experienced by the same plants) result in an increase or decrease in frost resistance respectively. *E.nitens* seedlings were significantly more frost resistant if grown at day/night temperatures of 12/8°C compared with 16/12°C (Table 5.5). However, substantial hardening only takes place with exposure to non-lethal temperatures ≤ 4°C for some part of the day and/or night. For instance, under controlled environmental conditions *E.nitens* seedlings taken from the glasshouse (25°C day/15°C night) hardened to about -7.5°C (from -3.2°C) over an eight week period if exposed to 25°C day/3°C night (Figure 5.3). Dehardening processes generally increase with increased day and/or night temperatures (Figure 5.4). However, both hardening and dehardening processes appear to respond to temperature in a quantitative rather than threshold manner. For instance, some hardening

takes place in *E.nitens* seedlings grown at day/night temperatures of 12/8°C (Table 5.5), whilst seedlings hardened to -7.5°C commenced dehardening when night temperatures were increased from 3 to ≥6°C (Figure 5.3, day temperatures and lighting also changed). The findings of other workers investigating physiological aspects of frost resistance in eucalypts generally support that found in *E.nitens*. Paton (1972) found that seedlings grown with 10°C nights were slightly hardier than those grown with 20°C nights (glasshouse conditions) and Paton (1972, 1980, 1981), Harwood (1980,1981) and Hallam (1986) all found that night temperatures of 4°C or lower are necessary to induce substantial hardening in eucalypts.

In contrast to the significant effects of temperatures on frost resistance, both hardening and dehardening responses are unaffected by photoperiod in *E.nitens* (Table 5.2, Figure 5.4). Although Eldridge (1969) reported a small increase in frost resistance of *E.regnans* seedlings grown under 8 h photoperiods compared with those grown under 16 h photoperiods (9°C/4°C day/night), Paton (1980) found no photoperiodic effects on frost resistance in *E.viminalis*. It therefore appears that the overall effects of photoperiod on frost resistance in *Eucalyptus* species are relatively small.

Another important physiological factor influencing the degree of frost resistance attained in *E.nitens* leaves is the interaction of root and shoot systems. Seedlings which had been hardened to -7.5°C and then exposed to constant 18°C shoot temperature dehardened at a lower rate if roots were kept at 3°C rather than at 18°C (Figure 5.4).

In *E.nitens,* one major difference between potted seedlings hardening under either natural or controlled environmental conditions, and planted trees hardening naturally is the greater level of maximum frost resistance which was generally measured in planted trees. In mid-winter, three year-old *E.nitens* hardened to T50 (frost temperature resulting in 50% leakage of cellular electrolytes) values of -10°C on average (Table 6.6), whilst potted seedlings reached T50 values of only -5.4 (Table 6.4a) and -8.0°C (Table 10.4) if naturally hardened (to early winter in Hobart) or artificial hardened (18°C day/2°C night) respectively. The greater hardening in plantation trees may possibly be due the effects of non-damaging sub-zero temperatures. Paton (1980) found subjecting already well hardened *E.viminalis* seedlings to artificial frosts (to -6.5°C) resulted in further increases in frost hardiness. Similar findings have been reported for *Pinus radiata* (Greer and Warrington 1982). The three year-old *E.nitens* progeny trial (Table 6.1) was subjected to more intense frosts than the potted seedlings hardening outdoors in Hobart, and the trees were more frost resistant than potted seedlings from identical families. The effects of non-damaging frosts on levels of frost resistance in *E.nitens* and other species could be examined using controlled facilities. Such techniques may reveal additional genetic variation in frost resistance. Another factor which may help explain these differences is the effects of plant size, age and/or development on frost resistance. In an *E.nitens* plantation, levels of late winter frost resistance were found to increase with increasing age of planting (Table 7.6). It may be that older trees, with presumably greater root systems and obviously greater shoot systems, may be able to produce more substances necessary for the metabolically active hardening process than the younger and smaller trees. Other processes (e.g. relative root growth) in younger trees may

demand a relatively higher portion of metabolites (at the expense of those available for hardening) than is the case in more established trees. These differences in relative frost resistance with plant size and/or age, together with the tendency for a greater part of the shoot of smaller trees to be in the coldest air layer {usually close to ground level, (Meskimen 1983)} may explain why new plantings are generally at a greater risk of frost damage than established trees.

The effects of plant size and or vigour on the development of frost resistance was also observed in potted *E.nitens* seedlings. In particular, there was a lack of substantial hardening in seedlings grown under constant 3°C (8 h day), compared with plants grown under identical night (3°C) conditions for 16 h and then 8 h of 13 or 25°C (Figure 5.3). Differences in relative frost resistance were not due to root temperatures and health *per se* (Table 5.3) and may possibly be due to relatively lower levels of metabolic activity in the seedlings grown under constant 3°C. Further evidence is provided by the findings that for the same families of *E.gunnii* and *E.nitens* X *E.gunnii* grown under identical conditions, smaller seedlings were on average 2.4°C less frost resistant than taller seedlings, after only four weeks hardening (Table 10.1).

Large differences have been demonstrated in the genetic ability of *Eucalyptus* species to develop high levels of frost resistance. In a relatively unhardened state (viz., summer) differences in relative levels of frost resistance between species may be about 2°C, but as they harden into winter some species harden to a much greater extent so that differences in relative frost resistance may exceed 7°C (Table 7.3). The maximum reported differences in winter frost resistance between the most sensitive and most resistant species is probably of the order *c.* 14°C (Martin 1948; Evans 1893). *E.nitens* is generally characterised by a moderately high level of frost resistance, and in winter is *c.* 5°C less resistant than the most resistant species (Table 7.3). The differences in relative frost resistance reported in this study are in good agreement with that reported elsewhere. In particular, *E.gunnii* has a high level of winter frost resistance (see also Martin 1948; Sakai *et al.* 1981; Destremau 1983 ; Evans 1983). Such differences in frost resistance between species are to a large extent genetically controlled. Using reciprocal grafts involving *E.gunnii* and the less resistant *E.nitens*, it was established that the frost resistance measured in juvenile foliage is dependent on the genotype of the foliage itself, irrespective of the genotype of the other part of the graft (Table 7.5).

Genetic variation in frost resistance within species has also been clearly demonstrated in this study for six of the 13 *Eucalyptus* species planted in the field trials, including *E.nitens* (Table 7.1). There may have been real differences in the frost resistance of the other seven species in the field trials that were unable to be elucidated by the natural frosts. Comprehensive studies with *E.nitens* identified two groups of provenances with different levels of frost resistance. Provenances from Southern N.S.W. and Errinundra showed less frost resistance than provenances from the Central Highlands of Victoria and Northern N.S.W. These two groupings were significantly different in unhardened (Table 6.3), partially hardened (Table 6.4a) and fully hardened (Table 6.4b) plants. This does not necessarily indicate that there may have been little interaction between group rankings and

level of hardiness, since large scale assessments were not made at all levels of hardiness, e.g. as plants dehardened. However, data collected from eight families (representing the full range of provenances) of *E.nitens* in a progeny trial,showed that as trees dehardened there were significant changes in the rankings of families from that established as they hardened (Table 6.6). This significant interaction between family and seasonal level of frost resistance has important implications for any breeding programme,where selection for superior frost resistance is highly desirable. In such cases, tree-breeders may have to decide at which times of the year the risk of frost damage is most critical,and make selections on the basis of assessments at those times. For example, if spring frosts rather than winter frosts are the major cause of concern, then screening of frost resistance may need to be made in spring rather than in winter.

Of the genetic variation in frost resistance in *E.nitens*, two independent assessments showed that 75% was attributable to differences amongst provenances,and the remainder due to differences within provenances (Table 6.5). Also, at well developed levels of hardiness, there was a consistent trend for increasing levels of frost resistance with increasing altitude of seed source within *E.nitens* provenances (Table 6.4). Indeed, this has been demonstrated for many *Eucalyptus* species {e.g. *E.regnans* (Ashton 1958; Eldridge 1969; Rook *et al.* 1980), *E.urnigera* (Thomas and Barber 1974), *E.pauciflora* (Pryor 1957a), *E.fastigata* (Sherry and Pryor 1967; Wilcox 1982c), *E.viminalis* (Paton 1972) and *E.delegatensis* (Hallam 1986)}. The general existence of this clinal variation in frost resistance is of potentially significant benefit to any programme of selection and breeding for frost resistance, because it means that gains should be realised by the careful selection of seed from colder sites (Pryor 1957a).

This study has demonstrated that genetic differences in frost resistance can be used to advantage in breeding programmes through controlled pollinations. This represents a more advanced level of breeding than selections of species and within species (op. cit. above). At this level of breeding, both interspecific and intraspecific controlled crosses were attempted. *E.nitens* was found to hybridize relative easily with species of close taxonomic affinity (species in the same series, viz., *Viminales*),and intraspecific crosses were generally easily produced (Table 8.3). However, the studies indicated that the success of controlled pollinations was dependent on the timing of emasculation and pollination (Table 8.1),and the compatibility of parents. In particular, some interspecific combinations appear incompatible, presumably because of vast differences in floral morphology (e.g. *E.globulus* X *E.nitens*, Table 8.2) and some intraspecific combinations display apparent self-incompatibility (Figure 8.5a).

Overall, morphological characters in both interspecific and intraspecific crosses displayed additive inheritance, although there was some evidence that a few characters appeared dominant (e.g. stem redness, development of lateral shoots). Hence, compared with both parents, progeny from crosses generally had intermediate morphology (Figures 10.2 to 10.4) and could be relatively easily identified, especially interspecific crosses.

In a similar manner the physiological character, frost resistance, appeared to display additive inheritance. For instance, after four weeks hardening the mean T50 values (frost

temperature resulting in 50% leakage of cellular electrolytes from leaf discs) of *E.nitens*, *E.nitens* X *E.gunnii* and *E.gunnii* were -5.3, -6.6 and -7.1°C respectively (Table 10.3). Within a specific hybrid such as *E.nitens* X *E.gunnii*, the pattern of additive frost resistance was consistent across a number of families. In a few cases, interspecific hybrids were only as frost resistant as the least resistant parent (e.g. *E.nitens* X *E.morrisbyi* and *E.nitens* X *E.globulus*, Figures 10.2a and 10.2b respectively). The frost resistance of progeny from intraspecific crosses generally displayed additive inheritance as well (Table 10.4). However, in one instance the progeny appeared to display heterosis, being 1.9°C more frost resistant than the hardiest parent after nine weeks hardening (Table 10.4). In this case the intraspecific cross was on average slightly more frost resistant than the *E.nitens* X *E.gunnii* families. Should this apparent heterosis be real, it offers much to selection and breeding efforts for frost resistance in *Eucalyptus* species, and in particular, *E.nitens*.

To accurately examine the inheritance of frost resistance in eucalypts there is need to have accurate estimates of parental frost resistance. In some instances, it may be possible to vegetatively propagate parents through grafting, cuttings and/or tissue culture. The *E.nitens* parental controls used in this study were mostly progeny from self-pollinations on the parents, and it is not known whether selfing may have depressing effects on levels of frost resistance as they have on growth in other *Eucalyptus* species (see Hodgson 1976c; Eldridge and Griffin 1983; Potts *et al.* 1987). Experiments should be designed to examine both the effects of self-fertilisation and vegetative propagation on frost resistance. These matters need to be addressed to make more definitive statements on the inheritance of frost resistance in eucalypts.

An holistic approach should be considered when selecting and breeding *E.nitens* and other genotypes for wood production in frost-prone environments. There are other factors apart from frost resistance which may be highly desirable in breeding programmes. These will undoubtedly include growth rate, and may also include tree form, wood quality and resistance to pathogens and insects. *E.nitens* provenances show considerable genetic variation in growth rate (Pederick 1979). Differences in frost resistance (this study) and growth rate (Pederick 1985) are also considerable at the family level. Early growth rate and frost resistance are largely uncorrelated in *E.nitens* (Figure 7.6) and it appears that both traits can be selected. Simultaneous selection for a number of traits has also been shown to be possible in *E.regnans* families (Wilcox 1982b).

12.2 Conclusions

Most early studies of frost resistance in eucalypts were made using field trials (Rook *et al.* 1980; Griffin *et al.* 1982b; Wilcox 1982c) or subjecting whole plants to either natural frosts (Eldridge 1969) or artificial frosts (Ashton 1958; Grose 1960; Paton 1972,1980,1981; Harwood 1980; Rook *et al.* 1980; Menzies *et al.* 1981). These studies largely concentrated on either genetic or physiological aspects of frost resistance. More recent studies have used tissue samples subjected to artificial frosts to assess physiological and/or genetical aspects of

relative frost resistance (Harwood 1981; Eldridge *et al.* 1983; Webb *et al.* 1983; Hallam 1986; Raymond *et al.* 1986).In this study all approaches were used to examine both genetic and physiological aspects of frost resistance in *E.nitens*, although greater emphasis was given to frost resistance determinations using tissue samples. These aspects were also examined in some other eucalypts. A number of findings bear significance, viz.,

(1) Frost resistance in *E.nitens* is physiologically controlled, largely by the temperature regime. Plants harden significantly when subjected to cold temperatures (*c.* 1.5 to 4°C) and deharden when exposed to temperatures warmer than those with which they were hardened. The attainment of substantial levels of frost resistance appears dependent on sufficient levels of metabolic activity in the plant.

(2) There is considerable genetic variation in frost resistance and growth within *E.nitens*, and between *E.nitens* and other *Eucalyptus* species. Genetic variation in frost resistance is generally most clearly expressed through different physiological capacities of genotypes to harden and deharden in response to changing environmental conditions (e.g. compared with *E.nitens*, *E.gunnii* hardens to much more frost resistant levels in winter than are evident in summer).

(3) Genetic differences in frost resistance appear largely heritable in an additive manner.

(4) There is need to integrate physiological and genetic aspects of frost resistance in eucalypts to more fully understand pattern of variation.

In frost-prone Tasmanian sites, *E.nitens* appears the most suitable eucalypt for plantation establishment, on all but the harshest sites. Even on sites where *E.nitens* suffers slight frost damage, its early growth is far superior to that of some more resistant species (Table 7.8). However, there is considerable genetic variation in frost resistance within *E.nitens*. The question arises, "Are the most resistant provenances being planted in Tasmania?" The Toorongo provenance is currently preferred in Tasmania. Although it is generally as frost resistant as the other provenances with the "juvenile-persistent form" from Victoria (i.e. Rubicon and Macalister), and the Northern N.S.W. provenance, its average level of resistance was slightly less than these other provenances, at both moderate and high levels of resistance (Table 6.4). Some of this overall lower resistance may be explained by the presence of some families with the "early-adult form" (generally less frost resistant) in experiments. Although seed currently used in A.P.P.M. plantations is generally collected from the Toorongo plateau, avoiding the "early-adult form", observations suggest that there is still some "contamination" with this genotype (*c.* 5%). Clearly, this should result in an overall net loss of production due to slower growth and lower frost resistance of the trees with the "early-adult form". There may be advantages in using alternative provenances with slightly higher frost resistance, but comparable growth and free from the "early-adult form", e.g. Rubicon. Another option would be to concentrate on selection of desirable families (even from different provenances) and to incorporate these into a breeding population. The ultimate strategie(s) will depend on the relative importance of frost resistance, growth and wood quality. The variations in growth and wood quality, particularly over rotation age, is still largely unknown.

It is conceivable that overall gains in productivity could be made on sites where *E.nitens* is damaged or killed by combining the rapid early growth of *E.nitens* and the superior frost resistance of species such as *E.gunnii* through hybridization. These hybrids should be capable of, firstly, surviving on some sites where *E.nitens* is killed by frost, and secondly, showing faster growth than other parental species, which are slower growing, yet more frost resistant. Additional gains could be made from selections from subsequent generations. Two alternatives are firstly, to try and select different species (e.g. *E.glaucescens*) which may be more frost resistant than *E.nitens*, though faster growing but less frost resistant than species such as *E.gunnii*, and secondly, attempting to extend the frost resistance of *E.nitens* by capturing any heterotic effects through controlled breeding. The relative gains of these different strategies is an area of research where future work could be directed.

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APPENDIX A

Details of *E.nitens* families (seedlots) used in frosting studies under controlled conditions and in field trials, at Hampshire and Racecourse. Abbreviations pertaining to source (Source Nº) are A- Associated Pulp and Paper Mills Ltd; N- New South Wales Forestry Commission Seed Store; S:- CSIRO Division of Forest Research Seed Centre; V:- Department of Conservation, Forests and Lands Victoria; and, W- Western Wildlife Supply.

Provenance Field Nº - Source Nº	Alt. (m)	Lat. (S)	Long. (E)	Location details
Northern New South Wales				
N01-W10	1450	31°50'	151°30'	Barrington Tops;Mt Carson
N02-W09	1450	31°50'	151°30'	Barrington Tops;Mt Carson
N03-W08	1450	31°50'	151°30'	Barrington Tops;Mt Carson
N04-W07	1450	31°50'	151°30'	Barrington Tops;Mt Carson
N05-W06	1450	31°50'	151°30'	Barrington Tops;Mt Carson
N06-W05	1450	31°50'	151°30'	Barrington Tops;Mt Carson
N07-W04	1450	31°50'	151°30'	Barrington Tops;Mt Carson
N08-W03	1450	31°50'	151°30'	Barrington Tops;Mt Carson
N09-W02	1450	31°50'	151°30'	Barrington Tops;Mt Carson
N10-W01	1450	31°50'	151°30'	Barrington Tops;Mt Carson
N11-A68	1480	31°50'	151°30'	Barrington Tops;Kholwa Trl.
N12-N10890	1450	31°50'	151°30'	Barrington Tops;Kholwa Trl.
N12-S12567	1500	30°29'	152°25'	Ebor;New England Nat. Pk (JD625) †
N13-A67	1480	30°29'	152°20'	Ebor;Majors Point
N14-S13378	1300	30°30'	151°05'	Ebor;Barren Mountain. (*11)
N15-S13281	1280	30°28'	152°15'	Ebor;Barren Mountain. (*12)
Southern New South Wales				
S01-S14437	1400	35°52'	149°30'	Tallaganda State Forest (MC283)
S02-S14437	1400	35°52'	149°30'	Tallaganda State Forest (MC279)
S03-S14437	1375	35°49'	148°31'	Tallaganda State Forest (WT05)
S04-S14437	1370	35°54'	148°30'	Tallaganda State Forest (WT03)
S05-S14437	1350	35°53'	149°30'	Tallaganda State Forest (WT01)
S06-S14438	1240	35°59'	149°34'	Badja State Forest (WT11)
S07-S14437	1230	35°52'	148°32'	Tallaganda State Forest (WT02)
S08-S14439	1190	36°36'	149°23'	Glenbog State Forest (WT12)
S09-S14438	1180	36°03'	149°33'	Badja State Forest (WT24)
S10-S14437	1180	35°49'	149°32'	Tallaganda State Forest (WT06)
S11-S14439	1100	36°37'	149°25'	Glenbog State Forest (WT15)
S12-S14438	1085	36°09'	149°31'	Badja State Forest (WT09)
S13-S14438	1060	36°06'	149°33'	Badja State Forest (WT23)
S14-S14438	1060	36°09'	149°30'	Badja State Forest (WT22)
S15-S14439	1060	36°36'	149°25'	Glenbog State Forest (WT16)
S16-S14439	950	36°36'	149°26'	Glenbog State Forest (WT18)
S17-A70	935	36°38'	149°26'	Glenbog State Forest
S18-S14439	900	36°10'	149°31'	Badja State Forest (WT21)
S19-S14439	900	36°34'	149°28'	Glenbog State Forest (WT19)
S20-S14439	900	36°36'	149°26'	Glenbog State Forest (WT17)

Errinundra

E01-A56	1125	37°18'	148°50'	Cobb Hill
E02-A55	1120	37°18'	148°50'	Cobb Hill
E03-A54	1075	37°18	148°49'	Errinundra road
E04-S12155	1070	37°12'	148°52'	Splitters Creek.
E05-V63	1050	37°18'	148°51'	Gunmark Range.
E06-S14440	970	37°09'	148°51'	Cottonwood Range. (WT34)
E07-S14440	960	37°09'	148°51'	Cottonwood Range. (WT35)
E08-S14440	935	37°10'	148°58'	Old Bonang-Bendoc Road. (WT36)
E09-A52	925	37°10'	148°50'	Clarkeville Road.
E10-V69	910	37°09'	148°50'	Cottonwood Range.
E11-S14440	820	37°14'	148°56'	Kellys Creek. (WT29)
E12-S14440	800	37°14'	148°56'	Kellys Creek. (WT28)
E13-S14440	790	37°14'	148°56'	Kellys Creek. (WT27)
E14-V68	760	37°18'	148°40'	Goongerah
E01-V67	760	37°18'	148°40'	Goongerah

Macalister

M01-V183	1280	37°31'	146°55'	Mt Wellington
M02-V184	1280	37°31'	146°55'	Mt Wellington
M03-V181	1280	37°31'	146°55'	Mt Wellington
M04-V40	1260	37°33'	146°28'	Connors Plain
M05-V186	1260	37°33'	146°28'	Connors Plain
M06-A62	1240	37°34'	146°30'	Spring Hill
M07-V49	1220	37°40'	146°30'	South Road
M08-A60	1200	37°43'	146°29'	White Star Mine Track
M09-A63	1170	37°32'	146°27'	Connors Plain
M10-V189	1160	37°26'	146°22'	Mt Skene
M11-A65	1120	37°28'	146°24'	Barkley River Road
M12-V46	1160	37°28'	146°24'	Barkley River Road
M13-V51	1100	37°43'	146°32'	South of Mt Useful
M14-V50	1100	37°43'	146°32'	South of Mt Useful
M15-A66	915	37°29'	146°24'	Mt Shillinglaw

Toorongo

T01-V174	1150	37°54'	146°21'	Mt Erica
T02-V171	1150	37°54'	146°21'	Mt Erica
T03-V115	1130	37°50'	146°21'	Mt St Gwinear
T04-V113	1130	37°50'	146°21'	Mt St Gwinear
T05-V120	1120	37°45'	146°17'	Marshall Spur
T06-A93	1040	37°47'	146°19'	Little Boys Creek
T07-A91	1040	37°50'	146°06'	Mt Mac Donald
T08-V651	1040	37°47'	146°19'	Little Boys Creek
T09-V116	1040	37°47'	146°19'	Little Boys Creek
T10-V675	1040	37°47'	146°19'	Little Boys Creek
T11-A92	860	37°48'	145°12'	Tanjil Bren
T12-V621	880	37°47'	146°06'	Plateau
T13-V620	825	37°47'	145°55'	Near Powelltown
T14-V619	825	37°47'	145°55'	Near Powelltown
T15-V122	850	37°47'	146°03'	Pennys Saddle
T16-A88	760	37°47'	145°04'	Toorongo River

Rubicon

R01-S12403	1160	37°22'	145°56'	Mt Torbrek (JD568)
R02-S12403	1160	37°22'	145°56'	Mt Torbrek (JD567)
R03-V20	1160	37°22'	145°56'	Mt Torbrek
R04-V18	1160	37°22'	145°56'	Mt Torbrek
R05-A79	1160	37°22'	145°56'	Mt Torbrek
R06-V13	1000	37°25'	145°48'	Tweed Spur
R07-A76	1010	37°24'	145°54'	Royston River Road
R08-V646	1000	37°25'	145°48'	Tweed Spur
R09-V14	1000	37°25'	145°48'	Royston Road
R10-V12	1000	37°25'	145°48'	Tweed Spur
R11-A75	940	37°23'	145°53'	Royston Road
R12-A73	940	37°22'	145°55'	Snobs Creek
R13-A72	940	37°22'	145°55'	Snobs Creek
R14-A71	940	37°22'	145°55'	Conn Gap Road
R15-V16	920	37°22'	145°55'	Snobs Creek
R16-S12404	610	37°22'	145°34'	Toolangi State Forest (*JD546) † †
R17-S12404	610	37°22'	145°34'	Toolangi State Forest (*JD547)
R18-S12404	610	37°22'	145°34'	Toolangi State Forest (*JD548)
R19-S12404	610	37°22'	145°34'	Toolangi State Forest (*JD549)
R20-S12404	610	37°22'	145°34'	Toolangi State Forest (*JD550)

†, individual tree identity
† †, these 5 seedlots combined in the field trials

APPENDIX B

Details of seed from 12 *Eucalyptus* species used in field trials (Chapter 7). Sources are ANM- Australian Newsprint Mills Ltd ; BP- Dr.B.M. Potts ; ND- Dr.N.J. Davidson ; RW- Mr.R.J.E. Wiltshire ; S:- CSIRO Division of Forest Research Seed Centre ; and WT ; the author, Mr.W.N.Tibbits. Provenances from which leaves were sampled for controlled frosting studies are indicated (†). The numbers in brackets following the location are the number of trees from which seed was collected. Individual tree identity was retained in seedlots, except where an asterisk preceeds the number in brackets.

Species	Source of provenance	Alt. (m)	Lat. (S)	Long. (E)	Location Details
subgenus *Monocalyptus*					
E.coccifera					
	WT †	1150	42°53'	147°13'	Mt Wellington, Tas.(6)
	BP	850	42°41'	146°38'	Mt Field, Tas.(4)
E.delegatensis					
	S13089 †	1220	41°31'	147°39'	Ben Lomond, Tas.(*9)
	S13096	460	41°28'	145°22'	Luina, Tas. (*5)
E.fastigata					
	S8587 †	1210	35°54'	149°48'	Oberon, NSW.(*8)
	S8589	910	37°08'	149°12'	Bombala, NSW.(*9)
E.fraxinoides					
	S13362 †	1250	35°57'	149°32'	Badja SF, NSW.(*6)
	S11939	650	35°00'	149°30'	Tallaganda SF, NSW(*12)
E.laevopinea					
	S11591 †	1160	32°00'	151°19'	East of Scone,NSW.(*?)
	S11653	1070	31°11'	151°26'	Walcha District, NSW.(*2)
E.regnans					
	S	1000	37°48'	146°30'	Mt Toorongo, Vic. (6)
	S †	650	42°47'	146°54'	Moogara, Tas.(6)
Subgenus *Symphyomyrtus*					
E.dalrympleana					
	S12097	1100	35°42'	148°50'	Cotter Hut, ACT (*2)
	WT †	450	42°13'	146°35'	Black Bobs, Tas.(5)
E.gunnii					
	BP †	1040	42°00'	147°46'	Shannon Lagoon, Tas.(6)
	ND	600	43°05'	147°10'	Snug Tiers, Tas.(6)
E.johnstonii					
	ANM	800	42°31'	146°30'	Misery Plateau, Tas.(*?)
	ND †	600	43°05'	147°10'	Snug Tiers, Tas.(6)
E.perrinianna					
	S12442	1555	36°22'	148°24'	Kosciusko Nat. Pk (*5)
	RW †	540	42°42'	146°39'	Strickland Road, Tas.(6)
E.rubida					
	S12089	800	35°40'	149°00'	Glendale Crossing, ACT(4)
	WT †	100	42°41'	146°50'	Karanja, Tas.(6)
E.urnigera					
	WT †	950	42°53'	147°14'	Mt Wellington, Tas.(6)
	ND	660	43°05'	147°10'	Snug Tiers, Tas.(6)

APPENDIX C

Relationship between thermohygrograph temperature and that of thermometers 10 cm above the ground, at Hampshire (▲) and Racecourse (△).

Thermohygrograph temperatures of 2 and 6°C, correspond to temperatures near ground of 0 and 4°C, respectively.